National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

University of Alberta

The response of Zea mays L. (maize) sperm cells to calcium

by

Connie Williams

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

IN

PLANT STRUCTURE AND DEVELOPMENT

DEPARTMENT OF BOTANY

Edmonton, Alberta

Spring 1996

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-10764-7

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR:	CONNIE M. WILLIAMS
TITLE OF THESIS	THE RESPONSE OF *ZEA MAYS* L. (MAIZE) SPERM CELLS TO CALCIUM
DEGREE	MASTER OF SCIENCE
YEAR THIS DEGREE GRANTED:	1996

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Connie Williams

Box 557
Raymond, Alberta
T0K 2S0

Date: Dec 22, 1995

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE RESPONSE OF *ZEA MAYS* (MAIZE) SPERM CELLS TO CALCIUM, submitted by Connie M. Williams in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE in PLANT STRUCTURE AND DEVELOPMENT

David D. Cass

David J. Gifford

Marek Michalak

Locksley E. McGann

Finding ways to be sure that
your ideas can flourish is necessary
if the flow of ideas is to be maintained.

James Fadiman

I would like to dedicate this work to the loving memory of my Mother, JoAnne LaRue Palmer Williams. I love you, miss you and will never forget you

ABSTRACT

To understand the effect of Ca^{2+} on *Zea mays* L. sperm cells, Ca^{2+} uptake kinetics were characterized in the presence or absence of various metabolic inhibitors and Ca^{2+} channel blockers and at various times after isolation. These results suggest that 1 hr old sperm cells have both active and passive transport of Ca^{2+}. The 12 and 24 hr old cells have a decreased ability to bind and internalize Ca^{2+}. These changes cause a decrease in internalized Ca^{2+} resulting in a decrease in Ca^{2+} uptake as compared to 1 hr old cells. SDS-PAGE gel profiles and their corresponding blots probed by barley calreticulin antibody, rabbit calreticulin antibody and pea calmodulin antibody indicates for the first time the presence of both proteins in higher plant sperm cells. Regulation was examined further by looking at the difference in protein phosphorylation patterns in the presence and absence of Ca^{2+}.

ACKNOWLEDGMENTS

I would like to express gratitude to my:

Supervisor: Dr. David Cass, for giving me the opportunity and freedom to conduct this research in the manner I thought was appropriate. I also appreciate his enthusiasm and participation during the course of my research.

Co-Supervisor: Dr. David Gifford, for his helpful and insightful suggestions, constructive criticisms and the use of his lab space.

Supervisory Committee:
Dr. Marek Michalak, for help with experimental design, use of his lab space, equipment and chemicals. He also generously donated the rabbit calreticulin antibody. I would also like to thank his technician Jody Groenendyk for all of her invaluable help.
Dr. Locksley McGann, for helpful suggestions and constructive criticisms of my research and manuscripts.

Faculty that was not on my committee:
Dr. Carol Cass, for her technical help on my uptake research as well as numerous discussions on the interpretation of results. I would also like to thank Karen King for her invaluable help showing me and working with me during the course of this work.
Dr. Aladar Szalay, for allowing me to work in his lab and lending me the assistance of his technical staff.

External references:

Dr. Peter Hepler (University of Massachusetts) for answering my inquiries
Dr. R.E. Williamson (Austrain National University) and Dr. F. Chen (Oregon State University) for giving me pea calmodulin antibody and barley calreticulin antibody. I would also like to thank them for answering my inquiries

Friends. who assisted me during my research: Duane Adams, Mark Bickers, Carmen Chubuk, Dr. Danilo Fernando, Doug Hogue, Janice King, Carol Kirk, John Laurie, Chen Liangfu, Jean Malcolm, Julie McDonald, Tito Migabo, Deloris Mowles, Robert Mullen, Peter Odiobala, Milada Selmer, Grace Siebor, Sandra Stone, Steven Williams, Rebecca Wrisko, Dr. Guichang Zhang

I would especially like to thank my family for their love and kindness, JoAnne Williams, Kenneth J. and Ginny Williams, Kenneth Jr., Melanie and Carson Williams, Brian, Launa, Nicole and Brett Williams, Patricia, Sheldon, Keli and Jessica Hatch, Jack, Shirley and Corrine Adams, Peter Odiobala, My four Grandmothers and my late Grandfather.

And my love and inspiration Duane Adams

Thank you all for your input and your support

Table of Contents

Chapter Page

1. INTRODUCTION 1

1.1 Effects of Ca^{2+} on Fertilization in Plants 3

1.2 The Role of Ca^{2+} in Mammalian Fertilization 5

1.2.1 Ca^{2+} Uptake 6

1.2.2 Ca^{2+} Modulated Proteins: Calreticulin and Calmodulin 6

1.2.3 Phosphorylation 7

1.3 Effect of Ca^{2+} on Maize Sperm Cells 8

1.4 The Present Study 9

1.4.1 Ca^{2+} Uptake 9

1.4.2 Ca^{2+} Modulated Proteins: Calreticulin and Calmodulin 10

1.4.3 Phosphorylation 12

2. MATERIALS AND METHODS 13

2.1 Chemicals 13

2.2 Isolation of Sperm Cells 13

2.3 Kinetic Studies Using The Oil Stop Techique 14

2.4 Measurement of Ca^{2+} Transport Using the Filter Method 15

2.5 Antibody Probing 16

2.6 Sperm Cell Protein Phosphorylation 17

3. RESULTS 18

3.1 Characterization of Calcium Uptake 18

3.2 Characterization of Binding 21

3.3 Effect of Metabolic Inhibitors on Ca^{2+} Uptake 23

3.4 Effect of Ruthenium Red on Ca^{2+} Uptake 23

3.5 Effect of Ca^{2+} Binding Inhibitors on Ca^{2+} Uptake 26

3.6 Effect of Inorganic Ca^{2+} Channel Blockers on Ca^{2+} Uptake 26

3.7 Effect of Monovalent Cations on Ca^{2+} Uptake 26

3.8 Effect of Organic Ca^{2+} Channel Blockers on

Ca^{2+} Uptake 30

3.9 Oil Stop Method versus Filter Method 30

3.10 Calmodulin 32

3.11 Calreticulin 32

3.12 Effect of Ca^{2+} on The Phosphorylation of Sperm Protein 36

3.12.1 Time Differences 36

3.12.2 Differences Between Gal-Mes and Ca^{2+} Treated cells 38

3.12.3 Differences Between EGTA Reaction Mixture and PS/IDS Reaction Mixture 38

4. DISCUSSION 39

4.1 Ca^{2+} Uptake 39

4.1.1 Kinetics of Ca^{2+} Uptake 39

4.1.2 Antagonists That Affect Ca^{2+} Transport 40

Inhibitors of Ca^{2+} Binding to Ca^{2+} Binding Proteins 40

Inorganic Ca^{2+} Channel Blockers 42

4.1.3 Antagonists That Do Not Affect Ca^{2+} Transport 43

Monovalent Cations 43

Organic Ca^{2+} Channel Blockers 44

4.1.4 Summary of Results 44

4.2 Ca^{2+} Binding Proteins **45**

4.2.1 Calmodulin **45**

4.2.2 Calreticulin **46**

4.3 Phosporylation **46**

4.3.1 18 kDa Phosphoprotein **47**

4.3.2 Is Other Protein Phosphorylation Stimulated by Ca^{2+} **47**

4.3.3 One Phosphoprotein Is Not Affected By The Presence Or Absence Of Ca^{2+} **48**

4.4 Possible Effect of Ca^{2+} On Maize Sperms **48**

4.5 Possible Roles of Ca^{2+} in Plant Fertilization **49**

4.6 Future Work **50**

4.6.1 Purification of the 18 kDa phosphoprotein **50**

4.6.2 Phospholipases and Proteases **51**

4.6.3 Determination of Ca^{2+} Levels in Maize Synergids And Pollen Tubes **51**

REFERENCES **52**

List of Figures

Figure		Page
Figure 1 A.	Time dependent increase in Ca^{2+} uptake	19
1 B.	Michaelis-Menton Kinetics of Ca^{2+} uptake	19
Figure 2	Uptake of Ca^{2+} into maize sperms at various times after isolation	20
Figure 3	Addition of A23187 to 1 hour old maize sperm	22
Figure 4	Effects of the metabolic inhibitor DNP on Ca^{2+} uptake into maize sperms	24
Figure 5 A.	Effect of varying concentration of RR on Ca^{2+} uptake into maize sperm cells	25
5 B.	Effect of RR on 1 and 24 hr maize sperm cells	25
Figure 6 A.	Effect of varying concentration of La^{3+} on Ca^{2+} uptake into maize sperm cells	27
6 B.	Effect of varying concentration of Gd^{3+} on Ca^{2+} uptake into maize sperm cells	27
6 C.	Effect of La^{3+} on 1 and 24 hr maize sperm cells	27
Figure 7 A.	Effect of varying concentration of Cd^{2+} on Ca^{2+} uptake into maize sperm cells	28
7 B.	Effect of Ni^{2+} on maize sperm cells	28

7 C. Effect of Cd^{2+} and Ni^{2+} on 1 and 24 hr maize sperm cells 28

Figure 8 Effect of monovalent cations K^+ and Na^+ on Ca^{2+} uptake into maize sperms 29

Figure 9 A. Effect of verapamil on Ca^{2+} uptake into maize sperms 31

9 B. Effect of nifedipine on Ca^{2+} uptake into maize sperms 31

9 C. Effect of diltiazem on Ca^{2+} uptake into maize sperms 31

9 D. Effect of bepridil on Ca^{2+} uptake into maize sperms 31

Figure 10 Pea Calmodulin antibodies are used to probe sperm cell extracts 33

Figure 11 Barley Calreticulin antibodies are used to probe sperm cell extracts 34

Figure 12 Rabbit Calreticulin antibodies are used to probe sperm cell extracts 35

Figure 13 Maize sperm phosphoproteins 37

ABBREVIATIONS LIST

ATP	Adenosine triphosphate
Cd^{2+}	cadmium ion
Ca^{2+}	calcium ion
CaM	calmodulin
CTC	Chlortetracycline
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
DNP	2, 4-dinitrophenol
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FDA	Fluorescene diacetate
Gd^{3+}	gadolinium ion
Gal	galactose
kD	kiloDaltons
La^{3+}	lanthanum ion
Mes	2-(N-morpholino)ethanesulfonic acid
Ni^{2+}	nickel ion
PMSF	phenylmethylsulfonyl fluoride
PS/IDS	Lαphosphatidyl L serine/Dioleoyl-sn-glycerol (C18:1, (cis)-9)
K^{+}	potassium ion

PEG	polyethylene glycol
PBS	phosphate buffer solution
Na^+	sodium ion
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	Tris[hydroxymethyl]aminomethane
TBS	Tris buffer solution
TTBS	Tween-20 plus TBS
RR	ruthenium red

1. Introduction

There is a substantial body of evidence that the majority of uni- and multicellular organisms require calcium for normal growth and function (Campbell, 1983). Sidney Ringer was the first person to investigate systematically the requirement of inorganic compounds for the normal function and survival of animal cells (Ringer, 1882; Ringer and Sansburg, 1894). He showed that calcium was necessary for the development of fertilizated eggs and tadpoles, and that calcium was important in the adhesion of cells to each other.

Ringer's experiments were followed by those of several other physiologists (for review see Berliner, 1933). They not only confirmed that calcium was necessary for maintaining the morphology and function of animal tissues, but also that calcium seemed to play a unique role in the action of stimuli such as, adrenaline and drugs such as digitalis (Berliner, 1933). Further evidence for the physiological importance of calcium came as a result of the development of tissue culture (Eagle, 1956). Calcium, sodium, potassium and magnesium are essential components of physiological saline solutions. Removal of calcium from tissue culture media results in changes in cell structure and a decrease in growth rate of cells (Shooter and Grey, 1952), as well as reducing the strength of adhesion between cells (Coman, 1954).

Plants also require calcium (Wyn-Jones and Lunt, 1967; Hepler and Wayne, 1985), concentrations in the range of 0.1 - 3 mM being normally required to maintain cell structure and function (Antonelli and Stadler, 1990; Morocz et al., 1990). Lack of calcium may result in changes in structure of intracellular organelles, a decrease in cell elongation, as well as affecting cell walls and the permeability of cell membranes to solutes and other ions. Plants have a special requirement of calcium during the onset of flowering (Hewitt and

Smith, 1974) and the nodulation of legumes following infection by *Rhizobia* (Dixon, 1969).

Ca^{2+} is known to function as a second messenger in animals (Campbell, 1983), but only recently has its importance in plants become appreciated (Hepler and Wayne, 1985). Ca^{2+} has been determined to be a second messenger for a variety of fundamental and specialized cellular activities in plant cells mediated between primary signals and the metabolic response (Hepler and Wayne, 1985). The general mechanism by which Ca^{2+} modulates responses to primary signals, such as, light, growth substances and gravity, is through changes in its concentration. It is thought that in plants cytoplasmic free Ca^{2+} concentrations are normally maintained at < 1 μM and that Ca^{2+} stimulated processes are activated by an increase in free Ca^{2+} concentration from 1 - 10 μM (Cohen, 1985; Kauss, 1987). This low Ca^{2+} concentration within the cell is maintained against high concentration of Ca^{2+} outside of the cell. At a higher concentration Ca^{2+} appears to function through calcium-binding proteins that have affinities for Ca^{2+} in the micromolar range. Calreticulin (Chen et al., 1994), calmodulin (Chandra et al., 1994; Griess et al., 1994; Jablonsky et al., 1991), calsequestrin (Xing et al., 1994), and calpollenin (Gong et al., 1993) are all Ca^{2+} binding proteins that have been found to occur in plants. The result of Ca^{2+} binding to a protein is usually a conformational change that causes activation and allows for the subsequent reactions that comprise the response. Such proteins have the ability to bind Ca^{2+} in a reversible manner with dissociation constants ranging from the nM to μM range under physiological conditions (Roberts et al., 1986). The magnitude of the response can be regulated by the concentration of Ca^{2+}. The Ca^{2+}-modulated protein may be enzymatic (example, Ca^{2+}-dependent protein kinase) or may be a regulatory protein, which in the presence of Ca^{2+}

binds to, and thereby regulates the activity of the appropriate enzyme. The altered enzyme then regulates some other cellular process (Kauss, 1987).

Ca^{2+} channels or pumps exist in the plasma membrane to maintain or alter Ca^{2+} concentration gradients. Two main types of Ca^{2+} transport mechanisms exist in higher plants to regulate intracellular Ca^{2+}. One is a Ca^{2+}-ATPase ('primary pump') which may be partially dependent on calmodulin and is believed to be primarily in the endoplasmic reticulum and plasma membranes (Kauss, 1987; Poovaiah and Reddy, 1987). This system has a very high affinity for Ca^{2+} and may be the primary pump which maintains low free cytoplasmic Ca^{2+} concentration found in unstimulated cells. The second type of Ca^{2+} transport pump ('secondary pump') utilizes proton gradients as the driving force and has been characterized as a Ca^{2+}/H^{+} antiport (Kauss, 1987). The Ca^{2+}/H^{+} antiport is found in the tonoplast and possibly the plasma membrane (Hepler and Wayne, 1985). Ca^{2+} transport systems in plastids and mitochondrial membranes are thought to regulate free Ca^{2+} concentrations within these organelles (Kauss, 1987).

1.1 Effects of Ca^{2+} on Fertilization in Plants

Fertilization involves the interaction and fusion between two haploid gametes, the sperm and the egg. Some of the most complex fertilization systems occur in flowering plants. The egg and sperm interaction is dependent on the operation of highly specialized dispersal systems, pollen-stigma interactions, regulation of pollen tube growth and complex cellular organization designed to deliever the male gametes to the female gametes. The complex series of specific interactions eventually leads to membrane fusion and the

delivery of the male genetic material to the egg. Calcium has been shown to have many roles in sexual reproduction in flowering plants. For instance, cytoplasmic Ca^{2+} has been strongly implicated in directing pollen tube growth (Malho et al., 1994). The pollen tube contains a Ca^{2+} gradient, the tip having high amounts of Ca^{2+} and regions further from the tip having lower amounts of Ca^{2+} (Pierson et al., 1994). Low Ca^{2+} concentrations surround the sperm cells in the growing pollen tube *in vitro* (Zhang et al., 1995). If the gradient is disturbed pollen tubes will not continue growth, but if the gradient is reestablished then the tube will begin to grow again (Miller *et al.*, 1992; Malho *et al.*, 1994; Pierson *et al.*, 1994). Some pollen tubes also grow towards elevated $[Ca^{2+}]$ (Steer & Steer, 1989). Pollen tube growth may be directed by high Ca^{2+} concentration found in the synergid (Chaubal and Reger, 1990, 1992). The pollen tube normally enters the embryo sac through the synergid (Huang and Russell, 1992a). Once the pollen tube is inside the synergid, its growth stops, the tip ruptures, and two sperm cells, a tube nucleus and some tube cytoplasm are released (Brownlee, 1994).

Embryo sac synergids of wheat and pearl millet exhibit a high concentration of Ca^{2+} (Chaubal and Reger, 1990, 1992). Some suggestions have been made as to the possible role of Ca^{2+} in the synergids. The Ca^{2+} may play a role in directing pollen tube growth (Sanders and Lord, 1992) or it may cause a cascade of events that lead to fusion of sperm cells with the egg and central cell (Brownlee 1994). It has been shown that high Ca^{2+} concentrations can cause fusion of maize sperm cells (Roeckel & Dumas 1993; Cass *et al.* (unpublished results)) and fusion of sperm and egg (Faure *et al.*, 1994). As sperm cells are released into the synergid where there is a high concentration of Ca^{2+} (Chabal and Reger, 1990, 1992), there may be a relationship between the high Ca^{2+} and physiological changes in the sperm cell. In mammalian systems

Ca^{2+} is essential to the activation and fusion of the sperm and the egg (Whitaker and Swann, 1993). It is possible that plant and animal systems may share some similar fertilization properties in regards to the role of Ca^{2+}.

1.2 The Role of Ca^{2+} in Mammalian Fertilization

In the female reproductive tract, mammalian sperm undergo a unique maturation step referred to as capacitation (Austin, 1951). Capacitation culminates in the ability of a sperm to undergo the acrosomal reaction upon coming into contact with the zona pellucida or cumulus oophorus (Florman and First, 1988). The acrosomal reaction allows the sperm to penetrate the zona pellucida and then fuse with the oocyte plasma membrane (Florman and First, 1988). Ca^{2+} is known to effect capacitation, the acrosomal reaction and fusion (Whitaker and Swann, 1993).

In mammalian fertilization, calcium appears to be essential for activation of both sperm and egg as well as sperm/egg binding (Whitaker and Swann, 1993), which involves stimulating phospholipase A_2, phospholipase C and proteases (Hinkovska-Galchev and Srivastava, 1992; Nagdas 1992; Fry et al 1992; Roldan and Fragio 1993). Action of these enzymes perturbs the plasma membrane which is necessary for acrosomal exocytosis. Mammalian fertilization appears to require; Ca^{2+} uptake, Ca^{2+} binding proteins (calmodulin and calreticulin) and some phosphorylation of proteins, to be successfully completed.

1.2.1 *Ca^{2+} Uptake*

Ca^{2+} uptake plays an important role in the sexual reproduction of mammals. Mammalian sperm require extracellular Ca^{2+}, some of which must be internalized, to become motile (Lindemann and Kanous, 1989), undergo capacitation and acrosomal reaction (Parrish et al., 1993). It has been suggested that Ca^{2+}-ATPase may play an important role during human sperm capacitation. A time-dependent decrease in endogenous enzyme activity would allow the intracellular Ca^{2+} concentration to rise to a critical value necessary for initiation of acrosomal exocytosis and subsequent successful fertilization (Dasgupta et al., 1994). The acrosomal reaction is dependent on internal Ca^{2+} concentration, it is initiated in sperm by ZP3 (zona pellucida binding protein 3), the stimulatory agonist in the egg's zona pellucida (Florman, 1994).

1.2.2 *Ca^{2+} Modulated Proteins: Calreticulin and Calmodulin*

Calmodulin and Calreticulin have been found in mammalian sperm cells (Hernandez et al., 1994; Ashizawa et al., 1994; Nash et al., 1994). It has been suggested that calreticulin and calmodulin participate in the acrosomal reaction based on their localization in the acrosomal region (Nakamura et al., 1993; Hernandez et al., 1994). A role for calmodulin has also been suggested during sperm/egg fusion (Courtot et al., 1994). The mechanism by which Ca^{2+} and the Ca^{2+} binding proteins regulate sperm cell functions has not been elucidated (Nakamura et al., 1993). It is possible that both animals and plants need calmodulin and calreticulin present during fertilization.

1.2.3 *Phosphorylation*

Protein phosphorylation and dephosphorylation has been strongly suggested to be involved in human sperm capacitation (Furuya et al., 1993). Several phosphoproteins have been detected in sperm from a variety of species (Huacuja et al., 1977; Chulavatnatol, 1982; Noland et al., 1984). However, few of these have been specifically identified and none have been rigorously associated with a specific function. Among those reported is a protein of molecular weight of about 55 kDa in the cytoplasm of bull sperm, the phosphorylation of which appears to be related to mobility (Brandt and Hoskins, 1980).

Although there is considerable evidence that protein phosphorylation plays a role in the initiation and regulation of flagellar motility (review by Lindemann and Kanous, 1989), little is known about the role of phosphorylation during capacitation and the acrosomal reaction. De Jonge et al., (1991 a, b) have provided evidence for the involvement of the diacylglycerol/protein kinase C pathway and the adenylate cyclase/cyclic adenosine 3':5' monophosphate (cAMP) pathway in the human sperm acrosomal reaction. Although the molecular mechanisms involved in the regulation of capacitation and the acrosomal reaction are still poorly understood, it is commonly accepted that protein phosphorylation may be associated with these events (Furuya et al., 1993). There are also 3 phosphoproteins (95, 51, 14 - 18 kDa) in human sperm that bind to zona pellucida (Naz and Ahmad, 1994), suggesting that phosphoproteins are involved in binding the sperm and egg together.

1.3 Effect of Ca^{2+} on the Maize Sperm Cells

Brewbaker and Kwack salts (BKS: 1.3 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.8 mM $MgSO_4 \cdot 7H_20$, and 1.6 mM H_3BO_3) have been used in protocols for isolation of maize sperm cells (Dupuis et al., 1987; Roeckel et al., 1988) and sperm cells from other species (review by Russell, 1991). Zhang et al. (1992a) examined the integrity and viability of maize sperms in the presence and absence of BKS, and showed that exposure of these cells to BKS results in a reduction in cell integrity. A new isolation and maintenance medium extending the life-span of isolated sperm cells has been developed based on the screening of six sugars, ten buffers and three membrane protective agents (Zhang et al., 1992b). Maize sperm cells can be maintained in culture for up to 72 hours (Zhang *et al.* 1992b). The viability of these cells was measured using FDA fluorescence and hemocytometry for morphological determinations. When sperm cells are exposed to Ca^{2+} (1 mM) they deteriorate very quickly (Zhang et al 1992b, 1995).

This loss of cell integrity may be an important part of the fertilization process. Pollen tubes of *Hordeum vulgare* and *Gossypium hirsutum* rupture and release their sperm into one of the synergids. Naked sperm nuclei enter the egg and the central cell (Jensen and Fisher, 1968; Cass and Jensen 1970; Mogensen, 1988). If this also occurs in maize, the sperm/egg and sperm/central cell membranes would fuse and sperm nuclei would be released into the egg and central cell just prior to fertilization. Ca^{2+} has been shown to play important roles in the adhesion and fusion of biological membranes (Leckband et al., 1993). The high sensitivity of the sperms to Ca^{2+} might be a factor which prepares sperm for fusion with their target cells Ca^{2+} may therefore play an important role in fertilization.

1.4 The Present Study

How does Ca^{2+} affect the maize sperm cell?

Ca^{2+} is known to play many roles in plant and animal fertilization. In the plant fertilization process the pollen tube grows until it reaches the synergid. Once inside the synergid, its growth stops, the tip ruptures, and two sperms, a tube nucleus and some cytoplasm are released. The sperms are released into the synergid which contains a high Ca^{2+} concentration (Chaubal and Reger, 1990, 1992). High Ca^{2+} concentrations cause maize sperms to lose membrane integrity *in vitro* (Zhang et al., 1995).

It is important to look at how the sperm deals with this high concentration of Ca^{2+}, if and how Ca^{2+} is getting across the membrane and how that Ca^{2+} might be affecting the physiology of the sperm. This can be done by looking at Ca^{2+} uptake, the regulation of Ca^{2+} uptake, Ca^{2+} binding proteins and the phosphorylation of sperm proteins in the presence and absence of Ca^{2+}.

1.4.1 *Ca^{2+} Uptake*

How is Ca^{2+} uptake achieved and how is it regulated?

Ca^{2+} cannot enter plasma membranes by simple diffusion: therefore ion carriers and ion channels are necessary to allow the movement of Ca^{2+} through the plasma membrane (Kauss, 1987; Hedrich and Schroeder, 1989; Jensen, 1981). Several types of Ca^{2+} channels have been demonstrated in the plasma membrane of higher plant cells (Schroeder & Hagiwara, 1990; Schroeder & Thuleau, 1991). Ion channels are proteinaceous openings that span the plasma

membrane and conduct ions on a concentration gradient (Hedrich and Schroeder, 1989). Ion channels also have specific receptor sites to which ion channel blockers attach (Hedrich and Schroeder, 1989; Bae *et al.*, 1989). Ion carriers are membrane-spanning ATPases (Hedrich and Schroeder, 1989).

Plant protoplasts allow study of solute movement across the plasma membrane without interference by the cell wall. Since flowering plant sperms are natural protoplasts (Russell, 1991) and can be harvested in very large numbers they represent nearly ideal cells for the study of ion fluxes, including Ca^{2+}.

1.4.2 *Ca^{2+} Modulated Proteins: Calreticulin and Calmodulin*

Are calmodulin and calreticulin present in maize sperm cells?

Ca^{2+} binding proteins calmodulin (CaM) and calreticulin are involved in regulating various cellular and biochemical processes. CaM is a key regulatory protein involved in Ca^{2+} signal transduction pathway of all organisms investigated to date. Calreticulin is a major Ca^{2+} storage protein in the lumen of the endoplasmic reticulum in animals (Michalak et al., 1992), it is also found in nonmuscle cells as well as many types of plant cells (Chen et al., 1994).

Ca^{2+} and CaM are involved in many physiological processes in animals and plant cells. These can be controlled by the level of Ca^{2+} (Blowers and Trewavas, 1988) or by the level of CaM (Cocucci and Negrinini, 1988). CaM is a small Ca^{2+} binding protein that has been highly conserved in animal and plant species (Roberts and Harmon, 1992) and is involved in mediating the Ca^{2+} response in cells. CaM has been identified, purified and characterized in a variety of higher plants, including zucchini, spinach, peanut, barley, oat, wheat

(Marmé, 1988), *Citrus sinensis* (Dubery and Schabort, 1987), avocado (Roux and Dubery, 1989), *Euphobia lathyris* and *Papaver somniferum* (Thompson et al., 1989), *Brassica campestris* (Lui and Du, 1989), maize (Griess et al., 1994) and *Arabidopsis thaliana* (Chandra et al., 1994). Plant enzymes which have been reported to be directly regulated by Ca^{2+} or Ca^{2+}-CaM include NAD-kinase, β-glucan synthase, Ca^{2+} and H^{+}-ATPase, quinate:NAD^{+}-oxidoreductase, and protein kinases (Poovaiah and Reddy, 1987)

Calreticulin is a major Ca^{2+} storage protein found in many animal species (Michalak et al., 1992; Milner et al., 1992) and in a few plants (Chen et al., 1994). In the smooth muscle sarcoplasmic reticulum and nonmuscle endoplasmic reticulum (ER) calreticulin is the major Ca^{2+} binding protein (Milner et al., 1991; Opas et al., 1991). In nonmuscle cells (Walz and Baumann, 1989) as well as in many types of plant cells (Hepler and Wayne, 1985), the ER is responsible for the regulation of cytosolic free Ca^{2+} concentration. Many cell types respond to external stimuli by elevating the cytosolic free Ca^{2+} concentration through the release of Ca^{2+} from an internal store.

Calreticulin is a Ca^{2+} binding protein involved in the release and sequestering of Ca^{2+} at a specific time. CaM is involved in mediating the response of Ca^{2+} within cells. It has been shown that in some plants the amount of free Ca^{2+} in the synergid before and after fertilization is different (Huang and Russell, 1992b). It has also been shown that after fertilization calreticulin increases within the ovary (Chen et al., 1994). CaM and calreticulin may be important in the fertilization process for binding, sequestering and release of Ca^{2+} or for the stimulation of a cascade of reactions involved in fertilization.

1.4.3 *Phosphorylation*

Phosphorylation is a method by which proteins are modified by the reversible covalent incorporation of a phosphate group. It is a widespread phenomenon with important consequences for metabolic control *in vivo*. The phosphorylation reaction is catalyzed by a protein kinase, usually in an ATP-dependent manner, whereas the reverse reaction is catalyzed by a phosphoprotein phosphatase. Each class of protein kinase is allosterically stimulated by specific 'signal metabolite' (i.e. cyclic nucleotides (cAMP or cGMP), diacylglycerol, Ca^{2+}) which makes enzyme phosphorylation responsive to extracellular signals. An increase in signal amplification can be made possible by designing an 'amplification cascade' with more enzymes involved (Cohen, 1983, 1985).

Phosphorylation may play an important role in signal amplification in maize sperms. What happens to the Ca^{2+} or what the Ca^{2+} does once it is inside the sperms may be important in how the sperms regulate external and internal Ca^{2+}. Ca^{2+} regulation was examined by looking at the pattern of phosphorylation in the presence and absence of Ca^{2+}.

During the course of fertilization the sperm cells are placed into the synergid. The synergid contains high concentrations of Ca^{2+}. How the sperm cell regulates this Ca^{2+} may be an important step to understanding some of the mechanics of sexual reproduction in flowering plants. In this study a few mechanisms that the sperms may use to regulate Ca^{2+} were explored. Ca^{2+} uptake, Ca^{2+} binding to Ca^{2+} binding proteins, phosphorylation and signal amplification could affect the regulation.

2. MATERIALS AND METHODS

2.1 Chemicals

$^{45}Ca^{2+}$ and [^{14}C]-PEG were obtained from Dupont (Wilmington, DE). [^{3}H]H_2O was obtained from Amersham Life Science (Little Chalfont, Buckinghamshire, England). The silicone-paraffin oil mixture was generously provided by C.E. Cass (University of Alberta, Edmonton, Alberta). The pea calmodulin antibody was generously provided by R.E. Williamson (Austrailian National University, Canberra, Austrailia). The barley calreticulin antibody was generously provided by F. Chen (Oregon State University Corvallis, Oregon). The rabbit calreticulin antibody was generously provided by M. Michalak (University of Alberta, Edmonton, Alberta). All other chemicals were obtained from standard commercial sources.

2.2 Isolation of Sperm Cells

Plants of maize (*Zea mays* L. cv 129 Crusader) were grown in controlled environment rooms (Zhang *et al.* 1992). Pollen was collected, sieved through 80 μm nylon mesh to remove shriveled pollen grains, and hydrated in a glass chamber in a moisture-saturated atmosphere at room temperature (22°C) for 20 min. Pollen was then immersed in a sperm isolation solution containing 0.55 M galactose and 2 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.7) in a ratio of 1 g of pollen per 10 ml of isolation solution and shaken at 130 rpm at room temperature (22°C) for 20 min. The burst mixture was sieved through 40 μm stainless steel filter to remove pollen exines. Sperm cells in the filtrate were purified using discontinuous Percoll (Sigma Chemical Co.) density

gradient centrifugation. Percoll density gradients (30, 15 and 10% in the isolation solution, pH 6.7) were prepared in 15 ml Corex centrifuge tubes. Five - 10 ml of the filtered sperm cell suspension was layered on top of the 10% Percoll and centrifuged at 3000 g for 60 min. Sperm cells, at the interface of 15 and 30% Percoll, were collected with a glass pipette, washed with the isolation solution, and centrifuged at 3000 g for 7 min. Approximately 3.5 x 10^6/ml were used. Cell viability was estimated using Evans blue dye exclusion. Morphologically normal cells were counted using a hemocytometer. Sterile technique was followed throughout. Sperm cells (100 μl) were placed in LB liquid broth and left for 2 days at 37°C to determine sterility.

2.3 Kinetic Studies Using The Oil Stop Technique

Calcium fluxes were measured at 22°C using an oil stop method protocol (Hogue and Cass 1994). One hundred μl of maize sperms (3 - 4 x 10^5 viable cells) were layered over 100 μl of silicone-paraffin oil mixture (sp. gravity; 1.032 g/ml; 84.9 parts Dow Corning 550 silicone oil plus 15.1 parts Fisher 0-119 light paraffin oil) in microcentrifuge tubes. The assay was initiated by addition of 100 μl of a $^{45}Ca^{2+}$ solution (0.55 M gal-mes and 122.1 kBq/ml $^{45}Ca^{2+}$) and terminated by centrifugation of cells through the oil layer. In determining the $^{45}Ca^{2+}$ content of cell pellets, supernatant fractions of assay mixtures were removed by suction and each tube was washed above the oil layer by addition of 1.5 ml of deionized water, which, together with most of the oil, was then removed by suction. To solubilize cell pellets, 0.25 ml of 5% Triton-X 100 was added to each tube. Total pellet H_2O and extracellular pellet H_2O volumes were determined by substituting $^{45}Ca^{2+}$ with [^{3}H] H_2O or [^{14}C] polyethylene glycol (PEG), respectively, and used for calculation of the $^{45}Ca^{2+}$ content of cells. Radioactivity associated with cell

pellets was determined by liquid scintillation counting. All assays were conducted in triplicate. Experiments in which the effect of Ca^{2+} channel blockers or inhibitors was investigated, the sperms were preincubated for 15 min. in the medium containing channel blocker or inhibitor before addition of $^{45}Ca^{2+}$. Ca^{2+} channels are blocked by lanthanum (La^{3+}), gadolinium (Gd^{3+}) and ruthenium red (RR) which all block Ca^{2+} binding to the Ca^{2+} binding proteins. These channels are also blocked by inorganic channel blockers, such as Ni^{2+} and Cd^{2+} and organic Ca^{2+} channel blockers such as verapamil, bepridil, diltiazem and nifedipine (Nayler, 1988; Bean, 1989). Inhibitors of energy metabolism (such as DNP) reduce ion uptake because of their effect on energy (ATP) availability for the process of transport or on the activity of the transport sites (Jensen, 1981). After centrifugation through the oil a control sample of cells containing no radioactivity were resuspended in 100 µl of gal-mes. These cells were counted and tested for viability.

2.4 Measurement of Ca^{2+} Transport Using The Filter Method

Accumulation of $^{45}Ca^{2+}$ was measured at 22°C in a reaction mixture containing 0.55 M galactose, 2 mM MES, 2 mM $CaNO_3$, pH 6.7 and $^{45}Ca^{2+}$ (approximately 122.1 kBq ml^{-1} in uptake medium). The reaction was started by addition of 500 µl of maize sperm cells (3 - 4 x 10^6/ml viable cells) suspended in 0.55 M galactose and 2 mM MES into 500 µl of reaction mixture. Duplicate 100 µl samples were removed at specific time intervals and filtered through Whatman (Maidstone, Kent, UK) WCN cellulose nitrate membrane filters (0.45 µm pore diameter). The procedure consisted of wetting the filters with 1 ml ice cold wash medium (PBS solution (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 150 mM NaCl, pH 7.4), rapidly filtering the sample under vacuum and rinsing with a

further 5 ml of wash medium (PBS). The filters were dried and the radioactivity was determined by liquid scintillation counting. Initial rates of Ca^{2+} uptake were measured by filtering the cells 15 sec after the addition of the $^{45}Ca^{2+}$. In experiments where the effect of channel blockers was investigated, the cells were pre-incubated for 15 min at 22°C with the inhibitor. Ca^{2+} uptake was initiated by the addition of the cells into the reaction mixture.

2.5 Antibody Probing

Protein profiles were obtained by one-dimentional SDS-PAGE using a 12% 0.75 mm gel (Laemmli, 1970) in the Mini-Protean II cell (Bio-Rad Labs Ltd.). Protein content was estimated according to Lowry et al. (1951). Following SDS-PAGE the gels were immersed for 20 min in chilled (4°C) transfer buffer (25 mM Tris, 192 mM glycine, and 20% [v/v] methanol). The subsequent electrophoretic transfer of proteins to nitrocellulose (Bio-Rad Labs Ltd.) was carried out overnight at 30 V (4°C) in a semidry Mini Trans-Blot Cell (Bio-Rad Labs Ltd.) according to the manufacturer's recommendations. All subsequent immunodetection procedures were carried out at 4°C with gentle shaking. Membranes were blocked in TTBS (500 mM NaCl, 20 mM Tris-HCL [pH 7.8] and 0.2% Tween-20) and 5% milk powder for 6 h, the membranes were washed once with TTBS, then incubated for 12 h with primary antiserum diluted (1:4000) in TTBS 1% milk powder. After washing for 1.5 h in several changes of TTBS, the membranes were incubated for 6 h with secondary goat anti-mouse (or goat anti-rabbit) IgG conjugated with horse radish peroxidase (Bio-Rad Labs Ltd.) dituted (1:3500) in TTBS in 1% milk. The membranes were washed for 1 h in several changes of TTBS and for 0.5 h in TBS (500 mM NaCl and 20 mM Tris-HCL [pH

7.8]). The ECL reaction was developed according to the supplier's instructions (Bio-Rad Labs LTd.) (Hogue personal communication).

2.6 Sperm Cell Protein Phosphorylation

The cells (3 - 4 x 10^6) were homogenized in 100 µl of homogenization buffer (625 mM Tris-HCL, 25 mM EDTA, 6.25 mM EGTA, 12.5 mM PMSF (Phenylmethylsulfonyl Fluoride), 12.5% triton X-100). The supernatant was divided in half and 27.5 µl of the supernatant was added to the reaction mixture (either 1) EGTA: 150 mM Tris, 10 mM $MgCl_2$, 5 mM EGTA, 8 µCi ^{32}ATP; or 2) PS/IDS (Lαphosphatidyl L serine/ Dioleoyl-sn-glycerol (C 18:1, (cis)-9)): 150 mM Tris, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM PS/IDS, 8 µCi ^{32}ATP), bringing the final volume of the reaction mixture plus supernatant up to 40 µl. The reaction mixture plus cell extract was incubated for 5 minutes at 30°C. The reaction was stoppped by adding 10 µl of a stop buffer (62.5 mM Tris-HCL pH 6.8, 0.1% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.125% Bromophenol blue) and boiling for 3 minutes in H_20 bath. The gel was loaded with approximately 40 µg of protein. Protein profiles were obtained by one-dimentional SDS-PAGE using a 7 - 20% gradient gel (Laemmli, 1970) in the Protean II xi cell (Bio-Rad Labs Ltd.). The gel was run overnight at 23°C. The ^{32}ATP gel was exposed to X-ray film and developed.

3. RESULTS

3.1 Characterization of Calcium Uptake

Ca^{2+} uptake in maize sperms increased in a time-dependent manner. An initial binding took place in the first 15 sec, followed by a linear uptake for approximately 30 min and then the uptake began to saturate (fig 1A). Approximately 60 - 70% of the uptake occurred in the first 20 min after initiation of uptake (fig 1A). Maximum uptake was observed at 30 - 60 min after isolation.

Ca^{2+} uptake in cells 1 hr after isolation yielded Michaelis-Menton (saturation) concentration-dependent kinetics (Fersht, 1985). Ca^{2+} uptake was characterized by a curve that approached saturation at the concentration of 1.0 mM (fig 1B). The data were obtained from Ca^{2+} concentrations between 0.001 - 10 mM in the first 10 - 15 min of uptake (linear phase of uptake). A Lineweaver-Burk plot of the kinetics of Ca^{2+} transport, done under linear conditions, gave Km and Vmax of 213 μM and 3.7588 nmol/μl cell H_2O/min respectively (fig 1B, *inset*). Ca^{2+} uptake initiated at different times (1, 3, 6, 12, and 24 hrs) after isolation displayed differences (fig 2). The rate of Ca^{2+} uptake achieved 1 hr after isolation is 2.682 nmol/μl cell H_20/min. Three hrs after isolation Ca^{2+} uptake was initiated, the rate of Ca^{2+} uptake is 2.037 nmol/μl cell H_20/min. Six hrs after isolation Ca^{2+} uptake was initiated, the rate of Ca^{2+} uptake is 1.536 nmol/μl cell H_20/min. Cells isolated 12 or 24 hrs before the initiation of Ca^{2+} uptake had a rate of uptake of 0.595 and 0.334 nmol/μl cell H_20/min, respectively, which is a decrease of 77% and 85% from the rate of uptake achieved with 1 hr after isolation cells. The cells isolated and incubated for 12

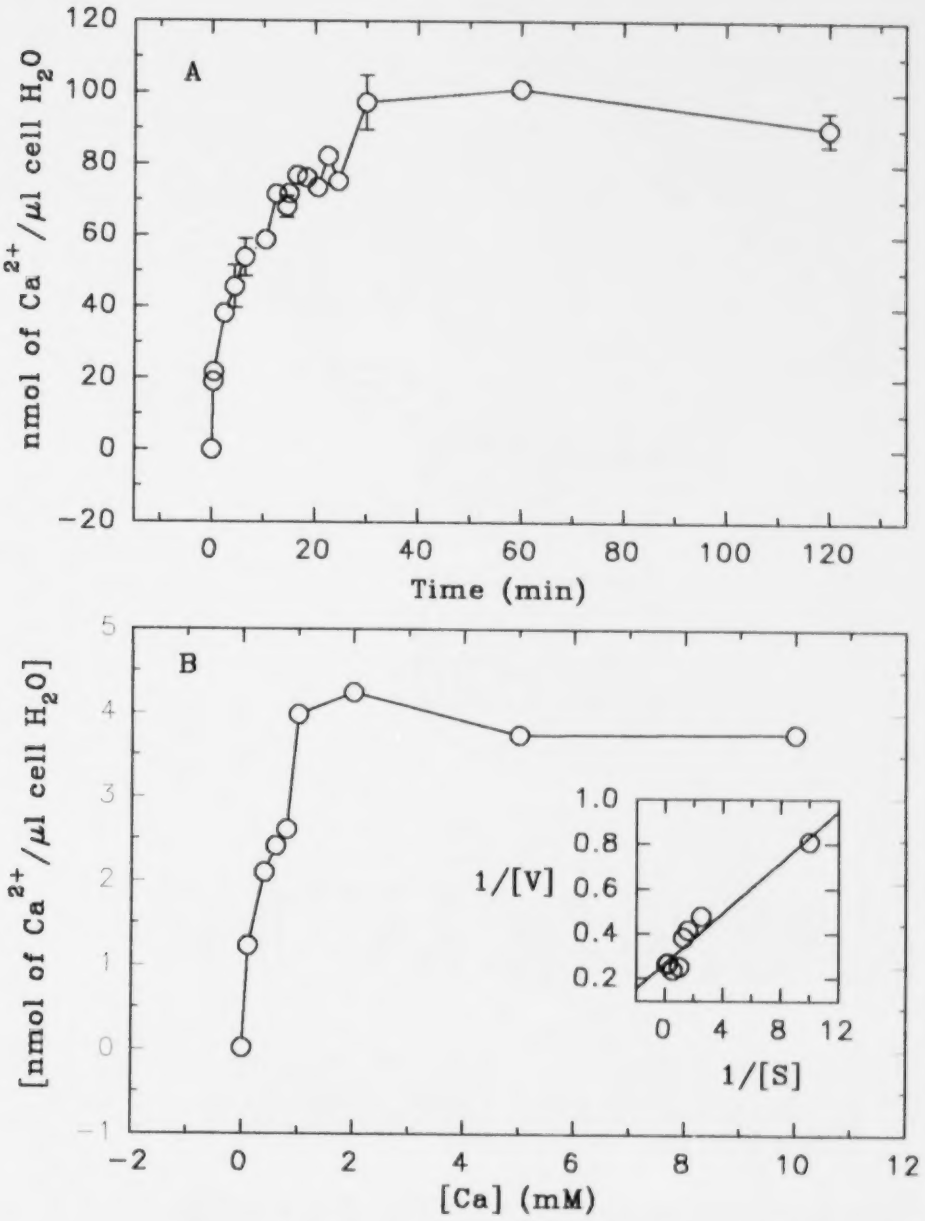

Fig. 1 A. Ca^{2+} uptake in maize sperms increased in a time dependent manner. The initial binding took place in the first 15 sec, followed by a linear uptake for the next 30 min and then saturation occurred.

B. Ca^{2+} uptake yielded Michaelis-Menton concentration dependent kinetics. Saturation of the curve was achieved at 1 mM. Km and Vmax are 213 μM and 3.759 nmol/μl cell H_2O. *inset*: Lineweaver-Burk plot of Ca^{2+} transport kinetics. (Oil Stop Method) Error bars represent ± SD (n = 3).

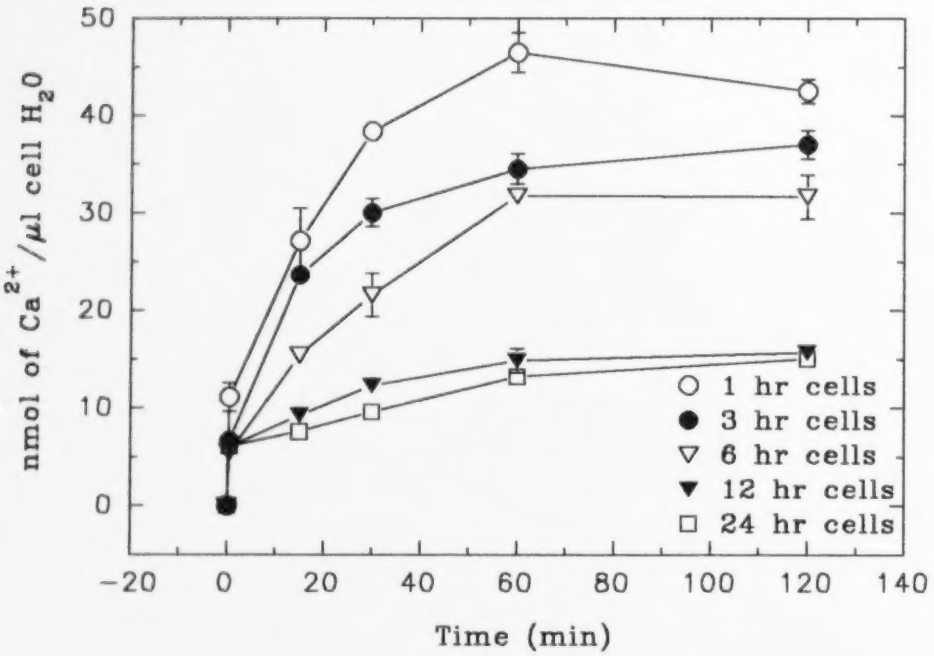

Fig. 2 Uptake of $^{45}Ca^{2+}$ into maize sperms various times after isolation. Ca^{2+} uptake initiated at different times after isolation was different. The decrease in Ca^{2+} uptake for 3 hr old cells was 24%, 6 hr cells was 43%, 12 hr cells were 77% and 24 hr cells were 85%. (Filter Method) Error bars represent ± SD (n = 3).

or 24 hrs before the initiation of Ca^{2+} uptake have some uptake but it is small compared to 1 hr cells. The rates of Ca^{2+} uptake are determined during the linear phase of the uptake.

3.2 Characterization of Binding

Transport of Ca^{2+} depends on 2 processes: 1) binding of Ca^{2+} to the transporter or channel, 2) internalization of the Ca^{2+}. The processes must be separated to determine what portion of the total $^{45}Ca^{2+}$ attributed to each. This can be accomplished with a Ca^{2+} ionophore A23187. The ionophore creates holes in the membrane which allows the free movement of Ca^{2+} into and out of the cell. A23187 enables the separation of the 2 processes involved in uptake of Ca^{2+}; by differentiating between the Ca^{2+} bound to the outside of the cell and the Ca^{2+} on the inside of the cell. When the cells are washed, the Ca^{2+} not bound to the cell (internally or externally) will be washed away. The only detectable $^{45}Ca^{2+}$ will be that which is bound to the cell. One hr old sperms incubated with A23187 before the initiation of Ca^{2+} uptake, performed similarly to sperms isolated 24 hour before the initiation of uptake (Fig 3).

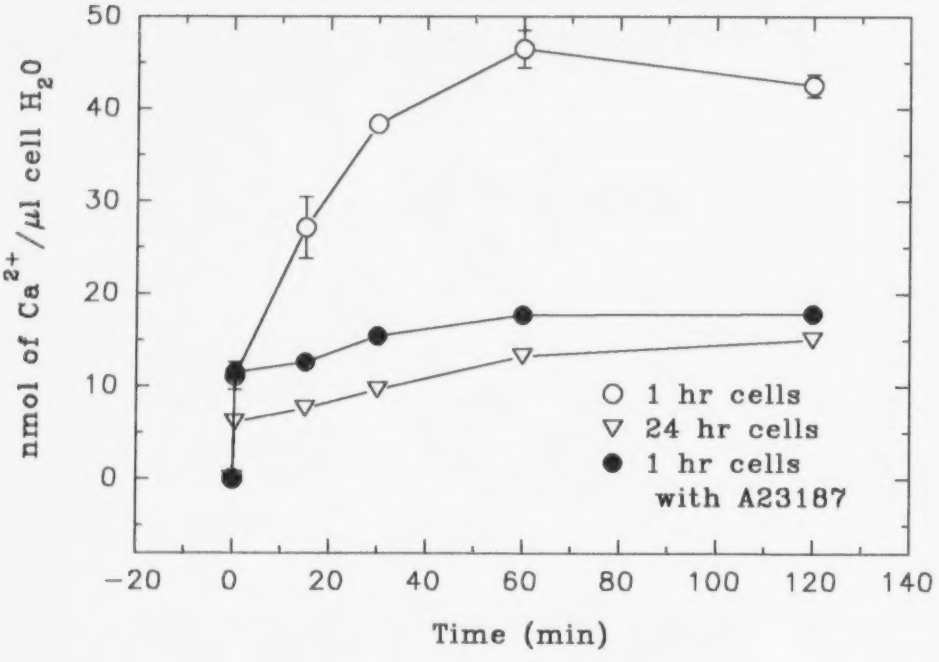

Fig. 3 Addition of A23187 (1μM) to the 1 hr maize sperms. The binding of Ca^{2+} to 1 hr cells is higher than to 24 hr cells. The kinetic results obtained using 1 hr cells in the presence of A23187 were similar to those obtained using 24 hr cells. (Filter Method) Error bars represent ± SD (n = 3).

3.3 Effect of Metabolic Inhibitors on Ca^{2+} Uptake

2,4-Dinitrophenol (DNP) has been used as an inhibitor of metabolic dependent uptake (Jensen, 1981). The presence of DNP in the uptake system would abolish the metabolic dependent uptake. Therefore, metabolic and non-metabolic components of uptake were separated by the addition of the metabolic inhibitor DNP. When 1 hr old sperms were pretreated with DNP (0.01mM) Ca^{2+} uptake decreased by 59% as compared with the control cells. This represents the active component of the net $^{45}Ca^{2+}$ uptake (Fig 4).

3.4 Effect of Ruthenium Red on Ca^{2+} Uptake

Ruthenium red inhibits the binding of Ca^{2+} to Ca^{2+} binding proteins (Charuk et al., 1990) and it also inhibits endomembrane Ca^{2+}-ATPases (Bae et al., 1989). When sperms were pretreated with RR (1.0 mM, 0.1mM, 0.01mM, 0.001mM) Ca^{2+} uptake decreased by 87%, 82%, 58%, 8%, respectively (Fig 5A). RR (0.1 mM) does not significantly affect 24 hour cells (Fig 5B). Most of the RR was excluded from control cells, but heat treated cells allowed the dye to enter. The majority of Ca^{2+} inhibition takes place on the outside of the control cells. Even at concentrations as low as 10 μM, RR inhibits Ca^{2+} binding to Ca^{2+} binding proteins, which decreases Ca^{2+} uptake into maize sperms. The RR inhibition we saw could not be due to interference with an endomembrane Ca^{2+}-ATPase because it did not enter the sperms. RR acted in a similar manner as Evans blue.

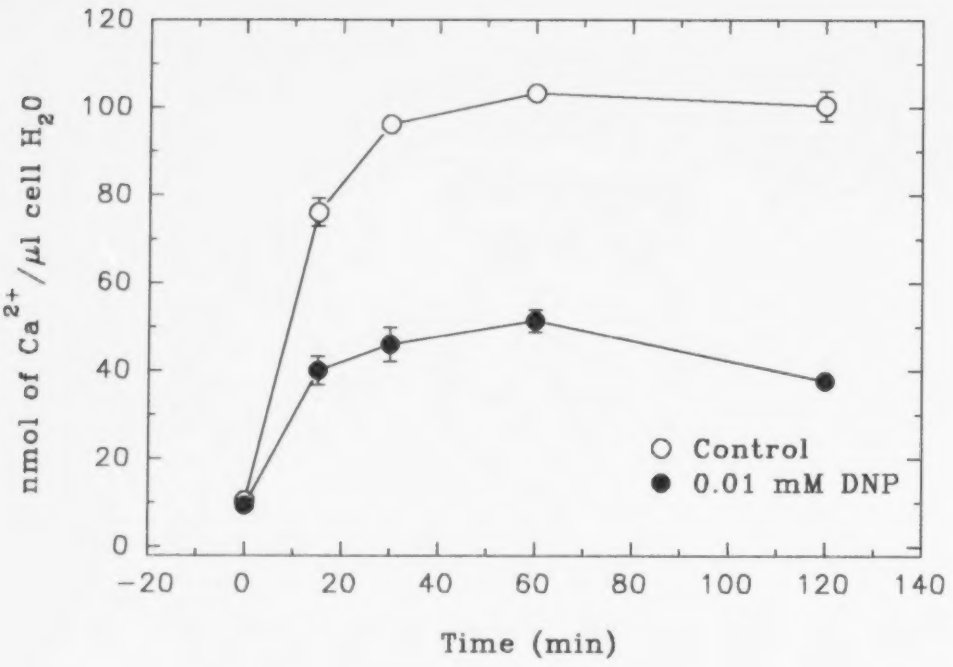

Fig. 4 Effects of the metabolic inhibitor DNP on Ca^{2+} uptake into 1 hr old maize sperms. The sperm cells were incubated with DNP for 15 min prior to initiation of uptake. DNP reduces the amount of Ca^{2+} uptake into maize sperm cells by 59%. (Oil Stop Method) Error bars represent ± SD (n = 3).

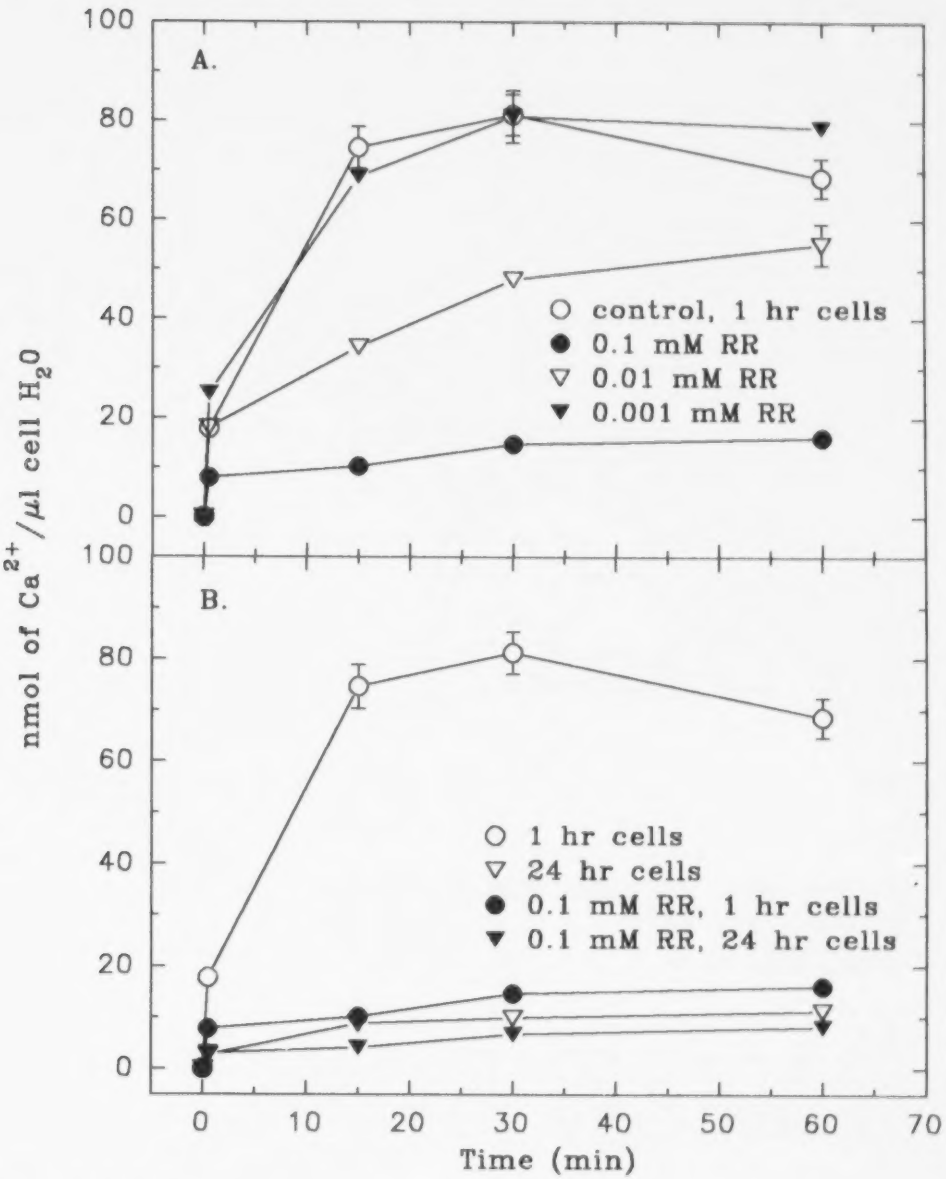

Fig. 5 A. Effects of ruthenium red (RR) on Ca^{2+} uptake into maize sperms. RR decreased Ca^{2+} uptake in a dose-dependent manner. Sperms preincubated in various concentration of RR 15 min prior to initiation of uptake. Decrease was observed with 0.1 mM RR (82%), and with 0.01 mM RR (39%) but no significant decreased was observed with 0.001 mM RR.

B. RR (0.1 mM) decreases Ca^{2+} uptake into 1 hr sperms by 82%. RR does not significantly decrease Ca^{2+} uptake into 24 hr sperms. (Oil Stop Method) Error bars represent ± SD (n = 3).

3.5 Effect of Ca^{2+} Binding Inhibitors on Ca^{2+} Uptake

It is probable that all membranes that bind Ca^{2+} also bind Lanthanide (Ln) ions (dos Remedios, 1981). Lanthanum (La^{3+}) and Gadolinium (Gd^{3+}) are two of the trivalent lanthanide (Ln) ions. Ca^{2+} and Ln have a similar ionic radii (dos Remedios, 1981) but the Ln ion has a higher charge density and therefore binds to Ca^{2+} binding sites with greater affinity than Ca^{2+} (dos Remedios, 1981). La^{3+} (1.0mM, 0.1mM, 0.01mM, 0.001mM) decreased Ca^{2+} uptake by 87%, 81%, 49%, 17% respectively (Fig 6 A). Gd^{3+} (0.1mM, 0.01mM, 0.001mM) decreased Ca^{2+} uptake by 85%, 49%, 32% respectively (Fig 6 B). La^{3+} (1.0 mM) does not significantly affect the uptake of Ca^{2+} into 24 hr maize sperm cells (fig 6 C).

3.6 Effect of Inorganic Ca^{2+} Channel Blockers On Ca^{2+} Uptake

Nickel (Ni^{2+}) and Cadmium (Cd^{2+}) can be used to distinguish between the different types of Ca^{2+} channels. T channels are blocked by Ni^{2+} (Hagiwara et al., 1988). Ni^{2+} (0.1 mM) caused a 81% decrease in $^{45}Ca^{2+}$ uptake (Fig 7 A). L and N Ca^{2+} channels are blocked by Cd^{2+} (Nowycky et al., 1985a, b). Cd^{2+} (1.0, 0.1, 0.01, 0.001) decreased $^{45}Ca^{2+}$ uptake by 87%, 82%, 58%, 50% respectively (Fig 7 B). Ni^{2+} (0.1 mM) decreases Ca^{2+} uptake into 24 hr cells by 36% (Fig 7 C). Cd^{2+} (1.0 mM) decreases Ca^{2+} uptake into 24 hr cells by 76% (Fig 7 C).

3.7 Effect of Monovalent Cations on Ca^{2+} Uptake

Studies of Ca^{2+} uptake in solutions containing up to 10 mM K^+ and 1 mM Na^+ indicated that the Ca^{2+} transport system was selective for Ca^{2+} over these

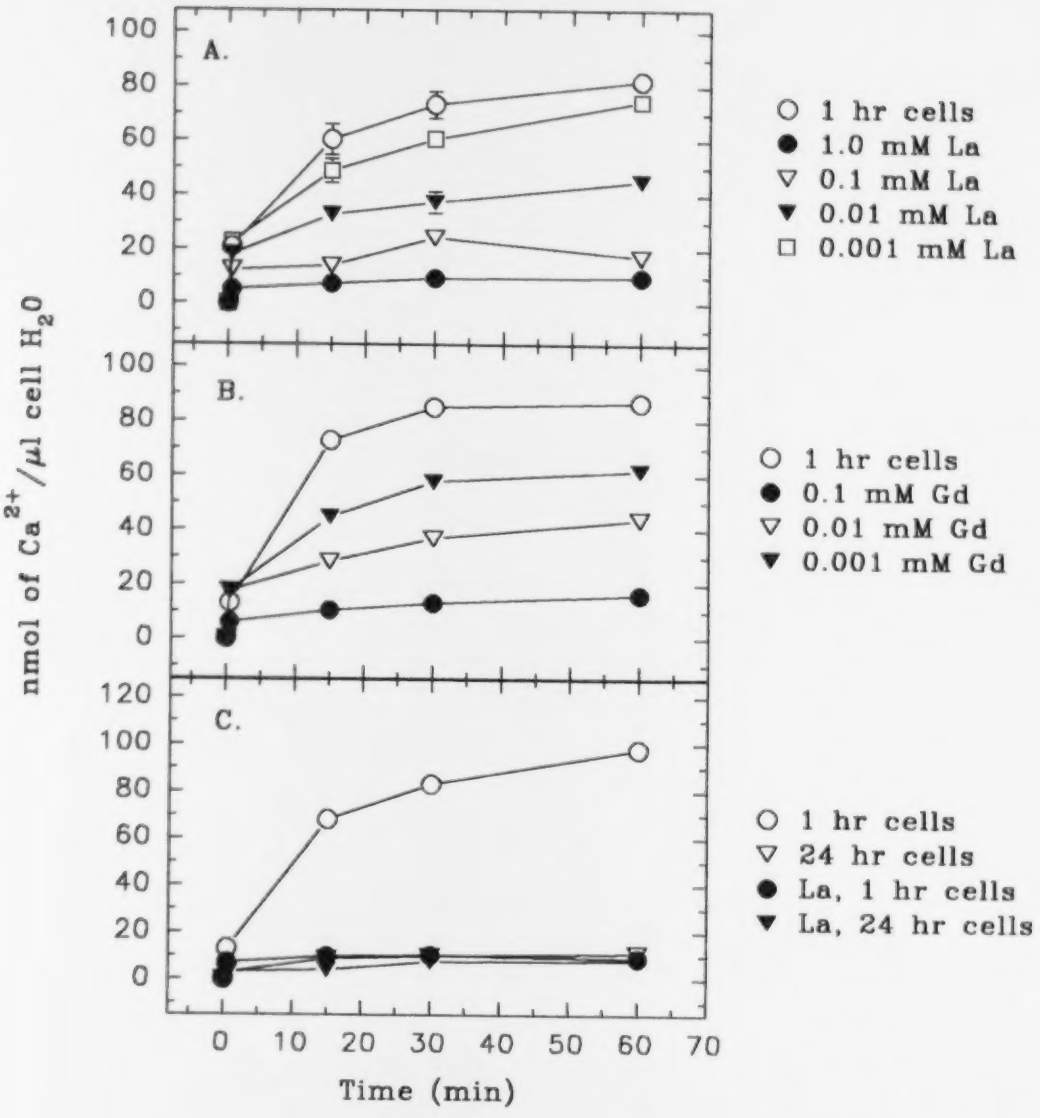

Fig. 6 A. La^{3+} decreased Ca^{2+} uptake in a dose-dependent manner. Decrease was observed with 1.0 mM La^{3+} (87%), 0.1 mM La^{3+} (81%), 0.01 mM La^{3+} (49%) and 0.001 mM La^{3+} (17%).

B. Gd^{3+} decreased Ca^{2+} uptake in a dose-dependent manner. Decrease was observed with 0.1 mM Gd^{3+} (85%), 0.01 mM Gd^{3+} (49%) and 0.001 mM Gd^{3+} (32%).

C. La^{3+} (1.0 mM) decreases Ca^{2+} uptake into 1 hr sperms by 88%. La^{3+} does not significantly decrease Ca^{2+} uptake into 24 hr sperms. (Oil Stop Method) Error bars represent ± SD (n = 3).

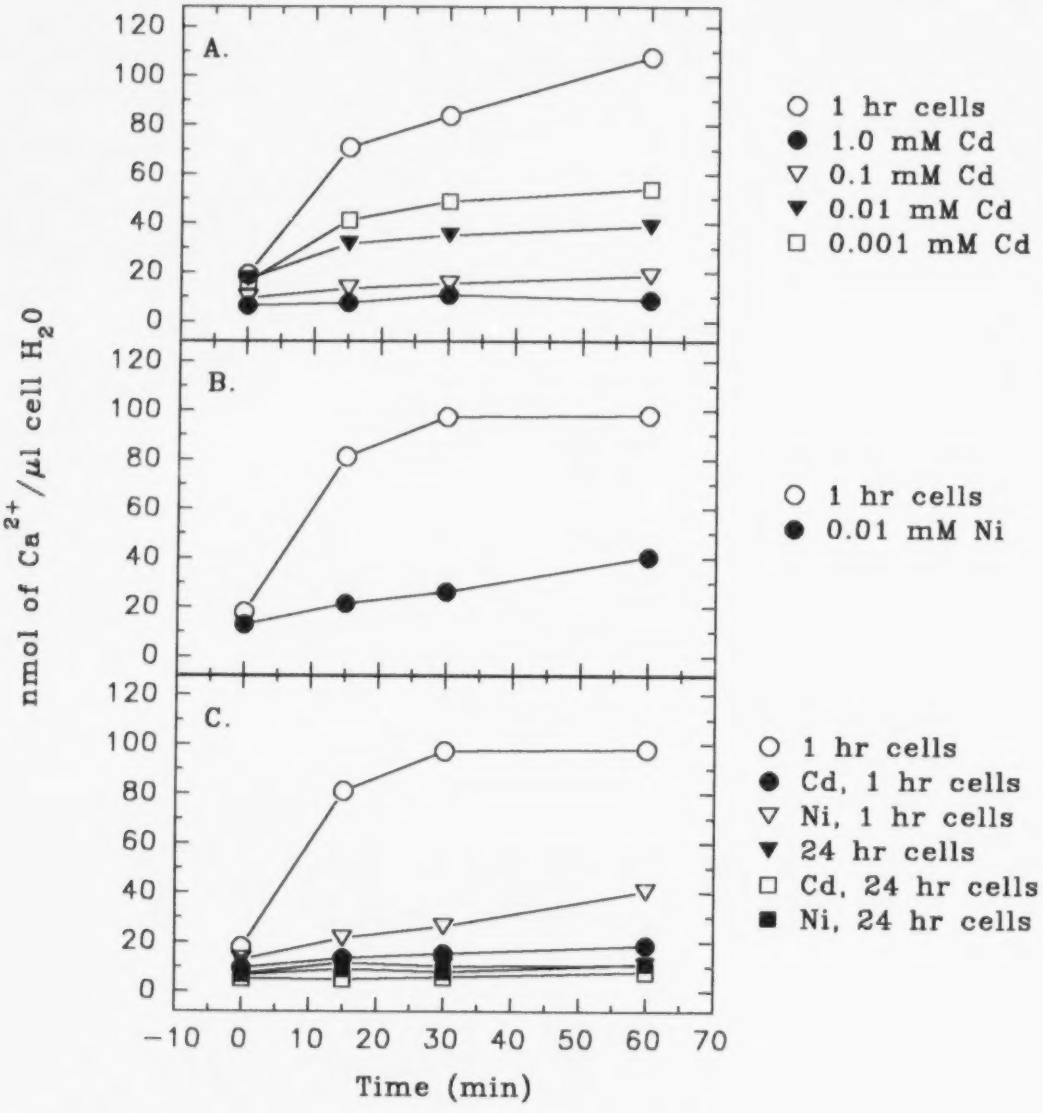

Fig. 7 A. Cd^{2+} decreased Ca^{2+} uptake in a dose-dependent manner. Decrease was observed with 1.0 mM Cd^{2+} (87%), 0.1 mM Cd^{2+} (82%), 0.01 mM Cd^{2+} (58%) and 0.001 mM Cd^{2+} (50%).

B. Ni^{2+} (0.1 mM) decreases the amount of Ca^{2+} uptake into sperms by 81%.

C. Cd^{2+} decreases the rate of Ca^{2+} uptake into 1 hr cells by 92% and decreases the rate of Ca^{2+} uptake into 24 hr cells by 76%. Ni^{2+} decreases the rate of Ca^{2+} uptake into 1 hr cells by 81% and decreases the rate of Ca^{2+} uptake into 24 hr cells by 36%. Error bars represent ± SD (n = 3).

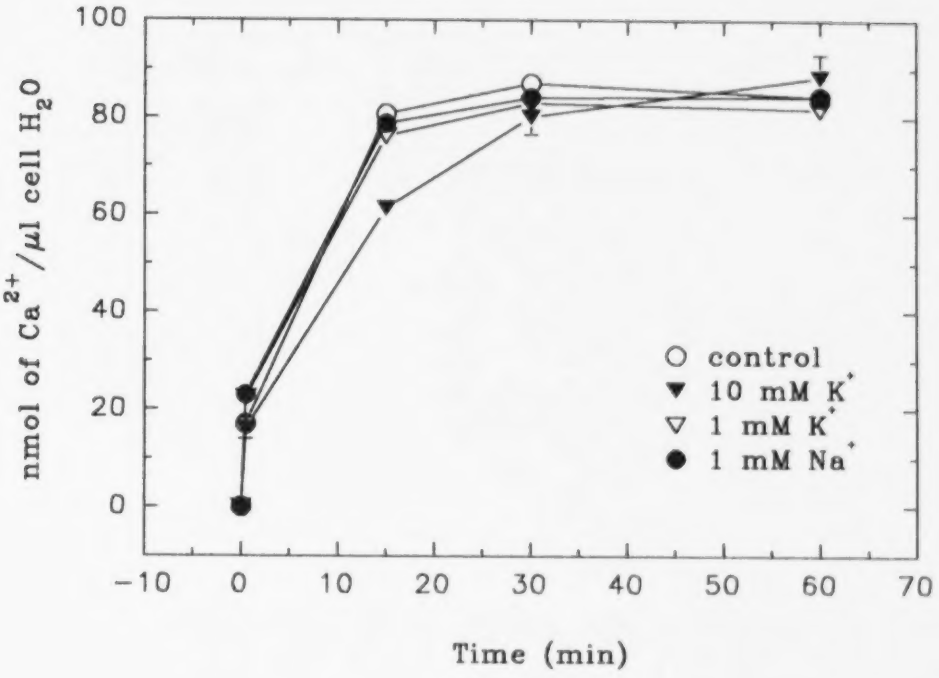

Fig. 8 Effect of monovalent cations on the uptake of Ca^{2+} into sperms. 10 mM and 1 mM K^+ and 1 mM Na^+ did not affect the uptake of Ca^{2+} into sperms. Error bars represent ± SD (n = 3).

monovalent cations (Fig 8). K^+ and Na^+ did not decrease the amount of Ca^{2+} uptake.

3.8 Effect of Organic Ca^{2+} Channel Blockers on Ca^{2+} Uptake

L -Ca^{2+} channel blockers such as verapamil (0.1 mM, 0.01 mM), diltiazem (0.1 mM, 0.01 mM, 0.001 mM), bepridil (0.01 mM) and nifedipine (0.1 mM, 0.01 mM), had little inhibitory effect on the rate of Ca^{2+} uptake (Fig 9 A, B, C and D). If uptake involved L-channels then it would be expected that these Ca^{2+} channel blockers would decrease Ca^{2+} uptake. Bepridil (0.01 mM) and verapamil (0.1 mM) caused a 20% decrease in cell number over the experimental period. Higher concentrations of verapamil, bepridil, nifedipine and diltaizem have been reported to decrease Ca^{2+} uptake in membrane vesicles (Harvey et al., 1989; Graziana et al., 1988). Maize sperms exhibit lower viability and ultimately died with increased concentrations of these organic Ca^{2+} channel blockers (based on Evans blue dye exclusion) (data not shown).

3.9 Oil Stop Method versus Filter Method

The Ca^{2+} uptake into maize sperms was higher during the oil stop method than it was during the filter method. This could be due to the fact that the filter method used suction and a high salt containing solution to wash the sperms. This could have caused some sperms to break or become damaged. This damage or breakage could cause a reduction in the total $^{45}Ca^{2+}$ associated with these cells. It was not possible to recover the cells from the filter to count them and assess their viability. The oil stop method may yield higher results because

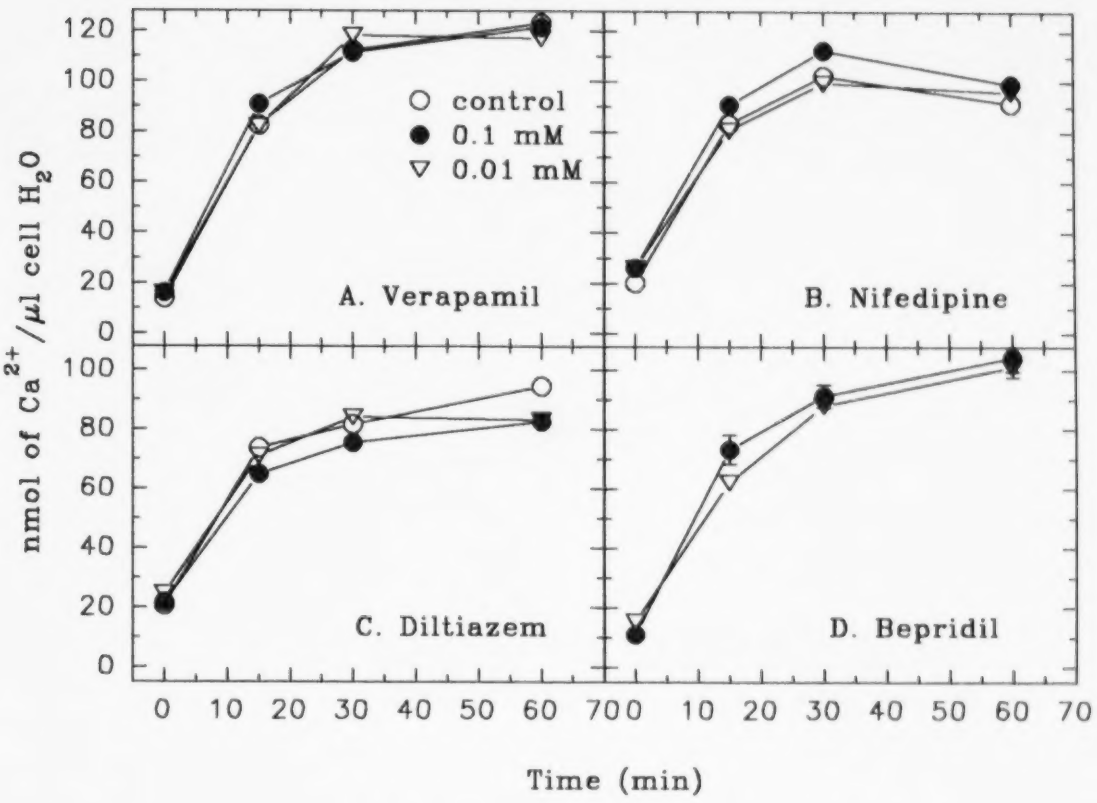

Fig. 9 Organic Ca^{2+} channel blockers do not affect Ca^{2+} uptake into maize sperm cells. A. Verapamil (0.1 mM, 0.01 mM) B. Nifedipine (0.1 mM, 0.01 mM) C. Diltiazem (0.1 mM, 0.01 mM) and D. Bepridil (0.01 mM) did not affect uptake of Ca^{2+} into sperms. Error bars represent ± SD (n = 5).

90% of the cells remained intacted and viable, whereas in the filter method this number could be lower.

3.10 Calmodulin

Ca^{2+} binding proteins calmodulin (CaM) and calreticulin are involved in regulating various cellular and biochemical processes. CaM is a key regulatory protein involved in the Ca^{2+} signal transduction pathway of all organisms investigated to date. Calmodulin was found to be present in all maize sperm cell extracts (Fig. 10). The amount of calmodulin present was not affected by the amount of time the cells were incubated. The cells incubated in Ca^{2+} seemed to exhibit more calmodulin than the cells incubated in gal-mes (fig. 10). The relative absorbance of the protein band from cells incubated in Ca^{2+} is 1.6 X greater than the absorbance of the protein band from cells incubated in gal-mes. Lanes a, c, e and g (Fig. 10) were proteins extracted from cells incubated in Ca^{2+}. These lanes show a slightly higher amount of antibody binding than the lanes b, d, f and h (Fig. 10) which were proteins from gal-mes incubated cells.

3.11 Calreticulin

Calreticulin is a major storage protein in the lumen of the endoplasmic reticulin in animals, it is also found in nonmuscle cells as well as many types of plant cells. Calreticulin was found in all cell extracts (Fig. 11 and 12). Exposing the sperm cells to Ca^{2+} for up to 24 hrs did not seem to affect the amount of calreticulin present. The rabbit calreticulin antibody (Fig. 12) has a lower binding affinity for maize sperm calreticulin than the barley calreticulin antibody (Fig 11).

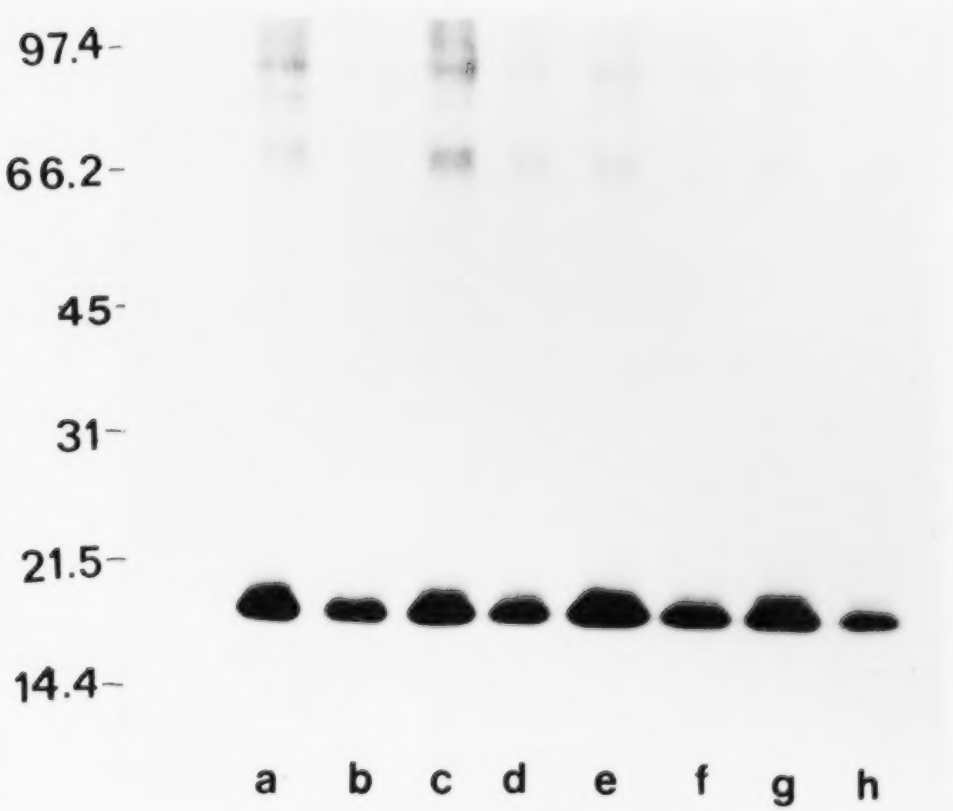

Fig.10 Pea calmodulin antibodies are used to probe sperm cells extracts. Cells were incubated in gal-mes or Ca^{2+} for various time periods and then the proteins were extracted. a. Ca^{2+} for 24 hrs b. gal-mes for 24 hrs. c. Ca^{2+} for 6 hrs. d. gal-mes 6 hrs. e. Ca^{2+} for 3 hrs. f. gal-mes for 3 hrs. g. Ca^{2+} 1 hr. h. gal-mes 1hr. Calmodulin from maize sperm cells is approximately 19 kD.

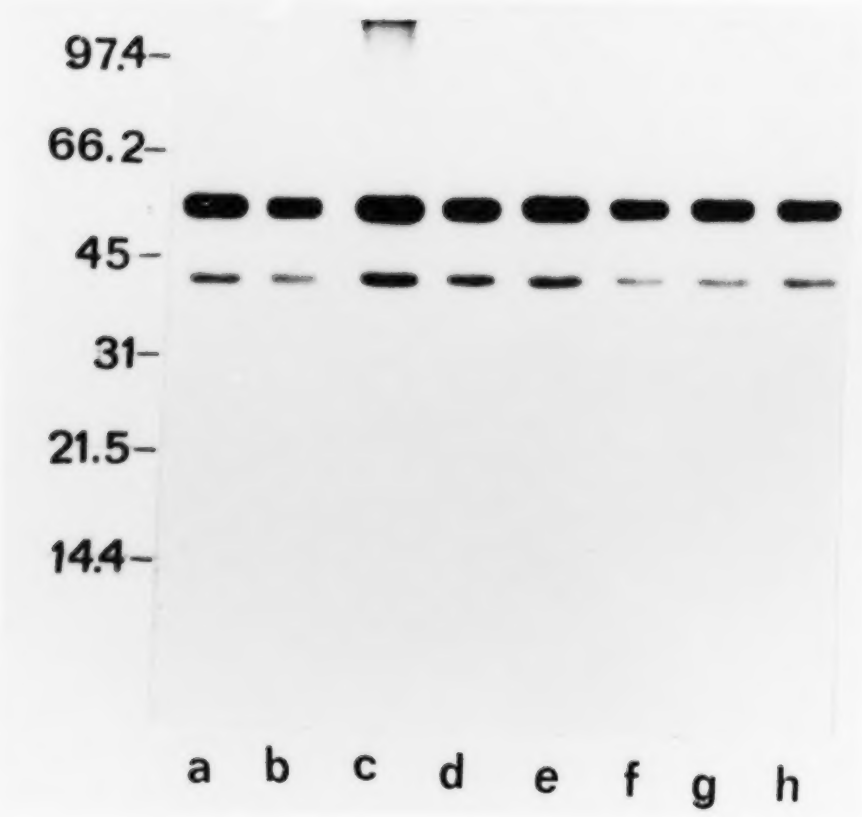

Fig.11 Barley calreticulin antibodies were used to probe sperm cell extracts. Cells were incubated in gal-mes or Ca^{2+} for various time periods and then the proteins were extracted. a. Ca^{2+} for 24 hrs b. gal-mes for 24 hrs. c. Ca^{2+} for 6 hrs. d. gal-mes 6 hrs. e. Ca^{2+} for 3 hrs. f. gal-mes for 3 hrs. g. Ca^{2+} 1 hr. h. gal-mes 1 hr. Calreticulin from maize sperm cells has the molecular weight of approximately 60 kD.

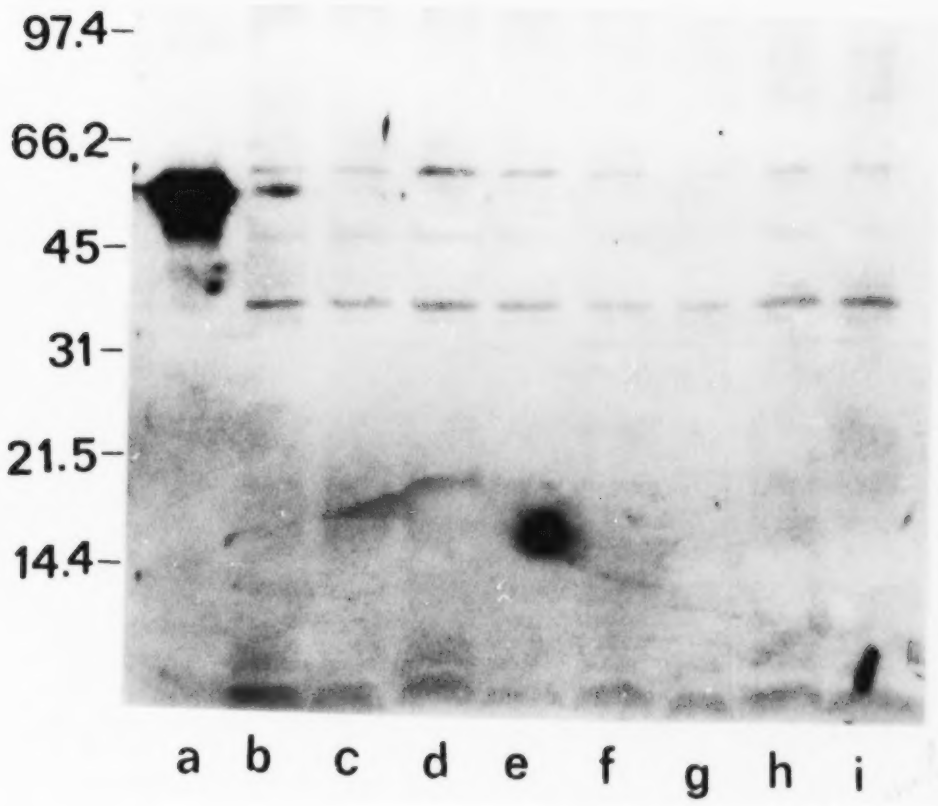

Fig. 12 Rabbit calreticulin antibodies were used to probe sperm cell extracts. Cells were incubated in gal-mes or Ca^{2+} for various time periods and then the proteins were extracted. a calreticulin protein from rabbit b. Ca^{2+} for 0.5 hrs c. gal-mes for 0.5 hrs d. Ca^{2+} for 1 hr e. gal-mes for 1 hr f. Ca^{2+} for 2 hrs g. gal-mes for 2 hrs h. Ca^{2+} for 3 hrs i. gal-mes for 3 hrs. Calreticulin from sperms has a molecular weight of approximately 60 kD.

The rabbit antibody recognized 2 proteins other than calreticulin, an approximately 45 kD and an approximately 40 kD protein. Plant and animal calreticulin have approximately 55% sequence homology (Chen et al., 1994). The proteins recognized by the rabbit antibody could be due to nonspecific binding. The barley antibody also recognized the 40 kD protein but this could also be due to nonspecific binding.

3.12 Effect of calcium on the phosphorylation of sperm proteins

3.12.1 *Time differences*

Maize sperms were exposed to Ca^{2+} or gal-mes for up to 24 hrs. These cells were then exposed to ^{32}ATP. The most dramatic increase in protein phosphorylation occurred in an 18 kD protein after 12 hrs of incubation (Fig. 13). This protein is apparent in freshly isolated cells, but the phosphorylation is very small. After 12 hr incubation there is a large increase in phosphorylation (Fig 13).

A 46 kD protein was strongly phosphorylated in freshly isolated cells but the phosphorylation decreased after a 1 hr incubation period. The phosphorylation of the 46 kDa protein began to increase in cells incubated for 12 hrs. The protein is strongly phosphorylated in cells incubated for 24 hrs (Fig 13).

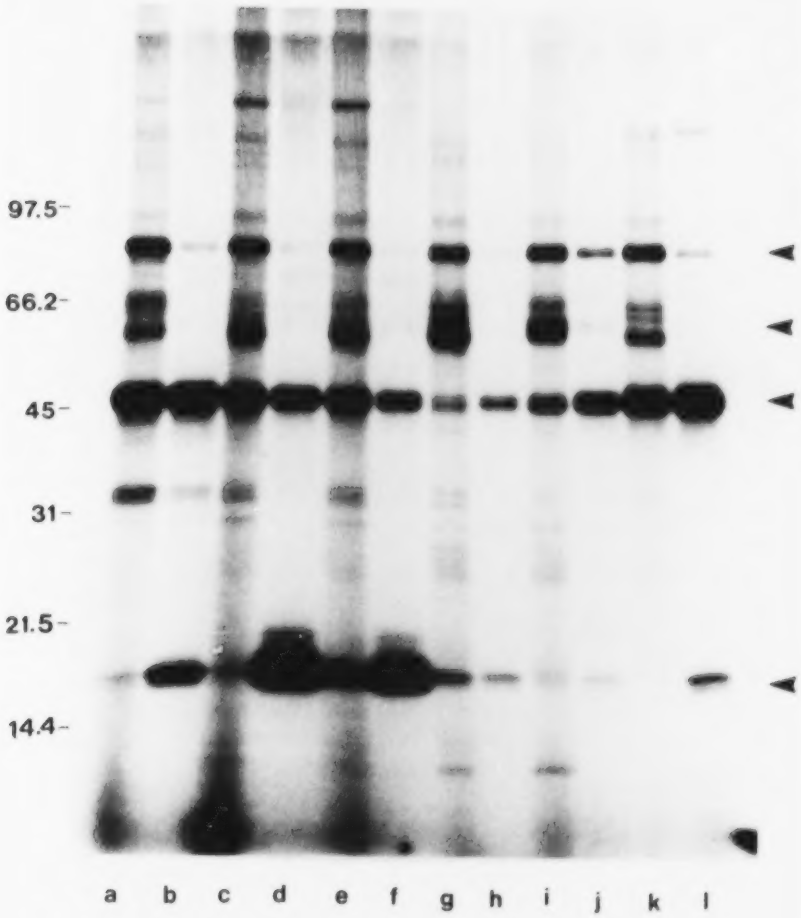

Fig. 13 Proteins were phosphorylated using ^{32}ATP. The cells (lanes a and b) were incubated in gal-mes for 24 hrs. The cell extract was then divided into 2, lane a: PS/IDS and lane b: EGTA. The cells (lanes c and d) Ca^{2+} - 24 hrs, lane c: PS/IDS and lane d: EGTA. Cells (lanes e and f) Ca^{2+} - 12 hrs, lane e: PS/IDS and lane f: EGTA. Cells (lanes g and h) gal-mes 1 hr, lane g: PS/IDS and lane h: EGTA. Cells (lanes i and j) Ca^{2+} 1 hr, lane i:PS/IDS and lane j: EGTA. Cells (lanes k and l) used immediately, lane k: PS/IDS and lane l:EGTA

3.12.2 *Differences Between Gal-Mes and Ca^{2+} Treated Cells*

The cells were incubated in either gal-mes or Ca^{2+}. The 18 kD phosphorylated protein was more apparent in the cells that had been incubated with Ca^{2+}. The cells that were incubated with gal-mes exhibited the protein but the phosphorylation was not as strong (Fig. 13).

3.12.2 *Differences Between EGTA Reaction Mixture and PS/IDS Reaction Mixture.*

Each cell batch from each incubation period was divided in half; one-half was exposed to a solution containing EGTA (Ca^{2+} chelating agent) and the other half to a solution containing PS/IDS (which stimulate the release of Ca^{2+} from its internal stores). There were 3 phosphorylated proteins close together with molecular weights between 57 - 66 kD and a fourth phosphorylated protein with a molecular weight of 84 kD. These 4 phosphorylated proteins appeared in the PS/IDS lanes and did not appear in the EGTA lanes. The 18 kD protein appeared stronger on EGTA lanes than on PS/IDS lanes.

Chapter 4. Discussion

4.1 Ca^{2+} Uptake

4.1.1 Kinetics Of Ca^{2+} Uptake

Ca^{2+} uptake in 1 hr old maize sperms increases in a time dependent manner. It is characterized by 3 phases: 1) initial binding, 2) linear uptake, and 3) saturation. These results indicate that the cells accumulate $^{45}Ca^{2+}$ and as the internal [Ca^{2+}] rises either the Ca^{2+} channels close or there is a significant Ca^{2+} efflux, resulting in Ca^{2+} flux equilibrium. The Km of 213 μM we obtained suggests the presence of a transporter in maize sperm cells. A similar Km value (167 μM) was obtained in membrane vesicles isolated from wheat roots (Huang et al., 1994). Transmembrane channels or pores found in vertebrates may be expected to have Km values in the high millimolar range and to be inhibited by known pharmacological Ca^{2+} channel blockers (Bean, 1989; Miller and Fox, 1990). Huang et al. (1994) determined that they were working with a voltage-gated Ca^{2+} transporter and that the voltage-dependent characteristics of Ca^{2+} transport were found to be similar to those exhibited by L-type Ca^{2+} channels in animals. They did not use any metabolic inhibitors in their study to determine if their uptake was an ATP dependent process. DNP (metabolic inhibitor) decreased the amount of Ca^{2+} uptake into maize sperms by approximately 59%, possibly indicating the amount of active transport (Jensen, 1981). Results from DNP and the kinetic studies on maize sperms indicate that a large proportion of Ca^{2+} uptake (approximately 60%) is due to an active transporter.

Cells, 12 and 24 hrs old exhibited decreased initial binding and saturation phases but not the linear phase of Ca^{2+} uptake. The decreased Ca^{2+} binding and

uptake phases in 12 and 24 hr old sperms might indicate that the mechanism by which Ca^{2+} is internalized is defective. This could be due to inefficient binding of Ca^{2+} to the channel or transporter and to deficient Ca^{2+} transport. The accumulation of internal Ca^{2+} could also be balanced by high activity of Ca^{2+} efflux which would explain the 24 hr results as well. Rat cells also exhibit some differences in Ca^{2+} permeability due to the aging of the cells (Higashino and Suzuki, 1994). The decreased internalization of Ca^{2+} displayed in 24 hr cells may also be due to ATP availability. When mung bean mitochondria are stored at +7°C for a few hrs they lose their ability to take up Ca^{2+} although no change in the respiratory control rate or membrane potential is observed (Åkerman and Moore, 1983). The uptake is restored again upon addition of Mg^{2+} and ADP (Åkerman and Moore, 1983).

4.1.2 Antagonists That Affect Ca^{2+} Transport

Ca^{2+} antagonists are used to characterize Ca^{2+} uptake across cell membranes. These antagonists function by modulating the movement of Ca^{2+} ions through Ca^{2+} selective channels (Nayler, 1988). The probable involvement of specific calcium channels may be demonstrated by application of calcium channel blockers.

Inhibitors Of Ca^{2+} Binding To Ca^{2+} Binding Proteins

Ruthenium red (RR) has been reported to affect Ca^{2+} transport. RR (0.1 mM) decreases Ca^{2+} uptake into 1 hr old maize sperm cells by 82%, but does not significantly inhibit Ca^{2+} uptake into 24 hr old cells. Most RR is excluded from maize sperms based on microscopy. It appears that most of the RR is

unable to cross the cell membrane, therefore most of the inhibition seen with RR is occurring on the outside of the cell. RR inhibits Ca^{2+} binding to Ca^{2+} binding sites on the Ca^{2+} channels and transporters on the outside of the the sperm cells. This mode of action occurs even at low concentrations (10 μM) of RR. RR has been used extensively as a rapid and sensitive method to detect all classes of Ca^{2+}-binding proteins in animal cells (Charuk et al., 1990; Sasaki et al., 1992). RR often displays higher affinity for Ca^{2+} binding proteins than Ca^{2+} does. One hr maize sperms display an inhibition of Ca^{2+} binding in the presence of RR and this inhibition is absent or very reduced in 24 hr old cells.

La^{3+} (1.0 mM) and Gd^{3+} (0.1 mM) cause a decrease in Ca^{2+} uptake into maize sperm cells as compared to control by 87% and 85% respectively. The decrease in uptake in the presence of La^{3+} and Gd^{3+} may result from inhibition of Ca^{2+} binding to the Ca^{2+} binding protein on the transporter or channel, or may also be due to the inhibition of voltage gated Ca^{2+} channels (Huang et al., 1994; Johannes et al., 1991). La^{3+} is a strong inhibitor of Ca^{2+} channel activity in animal cells (Kusaka and Matsushita, 1987; Reed and Bygrave, 1974) and has been shown to block Ca^{2+} influx into *Commelina communis L.* (Siebers et al., 1990), and *Zea mays L.* (Marshall et al., 1994) plasma membrane vesicles, into maize root segments (Rincon and Hanson, 1986) and into *Amaranthus* protoplasts (Rengel, 1994). At low concentrations La^{3+} may be the most effective Ca^{2+} channel blocker currently available for plants (Huang et al., 1994). RR, La^{3+} and Gd^{3+} all inhibit Ca^{2+} binding to Ca^{2+} binding proteins (Charuk et al., 1990; dos Remedios, 1981). Ca^{2+} uptake is effectively inhibited (87 - 85% decrease as compared to control) by these inhibitors in maize sperm cells, suggesting that internalization of Ca^{2+} into the sperm cell is dependent on binding.

La^{3+} has no significant effect on 24 hr cells. The 24 hr cells have a small amount of uptake occurring. This uptake is likely due to a different mechanism than the one blocked by La^{3+}. La^{3+} inhibits the binding of Ca^{2+} to the channel or transporter in 1 hr cells. These cells have inhibited binding in the presence of La^{3+} that is not present in 24 hr cells. This indicates that the 24 hr old cells are deficient in binding Ca^{2+} to facilitate its transport across the membrane. The decreased uptake reported in the present study may result from inhibition of Ca^{2+} binding to the Ca^{2+} binding protein on the transporter or channel. Our RR results also indicate that the decreased uptake in 24 hr cells results from a decrease in Ca^{2+} binding to Ca^{2+} binding protein on the transporter or channel. These results further indicate that internalization of Ca^{2+} into the sperm cell is dependent on binding.

Inorganic Ca^{2+} Channel Blockers

Can we distinguish among Ca^{2+} channels by using inorganic blockers? Ni^{2+} and Cd^{2+} decreased the amount of uptake into the maize sperm indicating the presence of Ca^{2+} channels. Micromolar concentrations of Ni^{2+} block T channels (Hagiwara et al., 1988) and Cd^{2+} blocks L and N channels (Nayler, 1988; Bean, 1989). Cd^{2+} causes a decrease in the rate of Ca^{2+} uptake into 1 hr sperms by 92% and a decrease in 24 hr cells by 79%. Ni^{2+} causes a decrease in the rate of Ca^{2+} uptake into 1 hr sperms by 81% and a decrease in 24 hr cells by 36%. This could indicate the presence of T, N or another type of voltage gated Ca^{2+} channels (Bean, 1989). Our results with Cd^{2+} might indicate the presence of a functional channel in the 24 hr cells. Ni^{2+} ability to inhibit uptake is diminished over time. This could indicate that the Ca^{2+} channels that Ni^{2+} inhibits are changing over time. This change might be due to an internal or external mechanism. It has been shown that the membrane lipid composition

can change over time (Chien et al., 1978; Verkleij and Post, 1987). This might cause a change in the protein/lipid interaction which might cause the ability of the channel to uptake Ca^{2+} to change.

4.1.3 Antagonists That Do Not Affect Ca^{2+} Transport

Monovalent Cations

High selectivity of Ca^{2+} over Na^+ and K^+ indicates that observed Ca^{2+} influxes were mediated by Ca^{2+} channels and not K^+ or Na^+ channels. The Ca^{2+} entry system, revealed by $^{45}Ca^{2+}$ flux measurements in maize sperms, was insensitive to variation of external K^+ and Na^+ concentration. Up to 10 mM K^+ and 1 mM Na^+ did not affect Ca^{2+} uptake into maize sperms. Voltage-gated channels are distinguished by virtue of their >1000-fold selectivity for Ca^{2+} over Na^+ or K^+, which greatly exceeds the selectivity of other Ca^{2+} entry pathways or other voltage-gated ion channels for their favored ion (Tsien and Tsien, 1990). High selectivity is not simply a matter of a close fit between the unhydrated Ca^{2+} ion and the pore since Ca^{2+} and Na^+ are nearly the same size (Tsien and Tsien, 1990). All Ca^{2+} channels use Ca^{2+} as their preferred charge carrier, and they are all insensitive to the traditional Na^+-channel blockers (for example tetraethylammonium (TEA)) (Nayler, 1988). Ca^{2+} channels from plasma membrane vesicles from wheat roots and carrot have also not shown sensitivity to Na^+ and K^+ (Huang et al., 1994; Graziana et al., 1988). Ni^{2+} and Cd^{2+} have been shown to block Ca^{2+} channels in maize sperms. These channels seem highly specific for Ca^{2+} because they are not affected by Na^+ or K^+.

Organic Ca^{2+} Channel Blockers

The apparent failure of organic channel blockers to inhibit Ca^{2+} uptake suggests the absence of L type channels in this cell type. In this study verapamil, diltiazem, bepridil and nifedipine did not affect $^{45}Ca^{2+}$ uptake into maize sperms. Right-side-out plasma membrane vesicles from maize roots were also unaffected by organic Ca^{2+} channel blockers (Marshall et al., 1994). In animal cells L channels are blocked by inorganic (e.g. Cd^{2+}) and organic (verapamil, diltiazem, nifedipine, bepridil) calcium antagonists (Nowycky et al., 1985a,b). Many studies have been done in plants using organic Ca^{2+} channel blockers. Most studies of Ca^{2+} uptake in higher plants have shown sensitivity to phenylalkylamines (e.g verapamil) and bepridil (Andrejavskas et al., 1985; Granziana et al., 1988; Harvey et al., 1989; Rengel and Elliott, 1992; Schroeder and Thuleau, 1991; Thuleau et al., 1993). Mosses and algae exhibit sensitivities to dihydropyridines or diltiazem (Conrad and Hepler, 1988; Dolle and Nultsch, 1988, MacRobbie and Banfield, 1988; Schumaker and Gizinski, 1993). In animal cells organic Ca^{2+} channel blockers are specific for Ca^{2+} channels when applied in submicromolar concentrations and at concentrations of 1 μM or greater they have nonspecific effects (Huang et al., 1994). In plant systems these compounds have effects when applied at 1 μM or higher concentrations (Huang et al., 1994). Inhibition by these organic channel blockers were studied in maize sperms using concentrations up to 0.01 - 0.1 mM; higher concentrations were not used because they damaged the sperms.

4.1.4 Summary of Results

In the present study La^{3+}, Gd^{3+} and RR were most effective at inhibiting Ca^{2+} uptake into 1 hr old cells followed by Cd^{2+}, Ni^{2+} and DNP. We also found

that K^+, Na^+, verapamil, diltiazem, bepridil and nifedipine did not significantly affect $^{45}Ca^{2+}$ uptake into maize sperms. The results from La^{3+}, Gd^{3+} and RR suggest that Ca^{2+} binds to the Ca^{2+} channel before it is internalized by the transporter or channel. This study suggests that Ca^{2+} crosses the cell membrane by both active and passive transport. Approximately 60% of the uptake is due to an ATP-dependent process. The Ca^{2+} channel is a T, N or another type of voltage gated Ca^{2+} channel, but the channel appears not to be an L- type Ca^{2+} channels in maize sperms. It was also shown in this study that there are some differences in the uptake of Ca^{2+} in cells of different ages. The 12 and 24 hr old cells have diminished ability to bind and internalize Ca^{2+}, resulting in a decrease in Ca^{2+} uptake as compared to 1 hr old cells.

4.2 Ca^{2+} Binding Proteins

4.2.1 Calmodulin

The monoclonal antibody raised to pea calmodulin (CaM) was used to detect calmodulin in maize sperm. This was the first time that CaM was identified in sperm cells. CaM has been found to be present in many plant cells (Marmé, 1989). It is present in the tapetal cells during anther development (Willemse, 1993). Tapetal cells are thought to store and supply calcium during anther develpoment (Willemse, 1993). Protein extracts from *Brassica campestris* pollen (Lui and Du, 1989) also showed the presence of CaM. CaM may play an important role during pollen development and regulating pollen tube growth (Lui and Du, 1989). It may also be important in other areas of sexual reproduction in flowering plants, like fusion or sperm activation. Ca^{2+}-CaM may

prove to be important in stimulating some kinases involved in sperm action. CaM may also regulate Ca^{2+}-ATPase, which is important in regulating intracellular Ca^{2+} levels. CaM may prove to be important in regulating intracellular Ca^{2+} mediated events within the sperm cell.

4.2.2 Calreticulin

Antibodies raised to rabbit and barley calreticulin recognized maize sperm calreticulin. The barley antibody recognized maize calreticulin with a higher affinity than did the rabbit antibody. The barley antibody did not recognize calreticulin isolated from rabbit. The similarity between animal and plant calreticulin gene sequences is 55% (Chen et al, 1994). It is therefore possible that the barley antibody could be raised to an epitope on calreticulin that the rabbit does not contain. This could explain why the barley antibody recognizes the maize sperm calreticulin more efficiently than the rabbit antibody does. It might also explain why the barley antibody does not recognize rabbit calreticulin. Calreticulin may prove to be important in Ca^{2+} binding, storage and release within the maize sperm cells.

4.3 Phoshorylation

Differences occurred when the cells were incubated over time and in the presence and absence of Ca^{2+}. There were also differences seen when the supernatant from the incubated sperms were divided in half and exposed to either EGTA (Ca^{2+} chelating agent) or PS/IDS (which stimulates the release of Ca^{2+} from its internal stores).

4.3.1 18 kDa Phosphoprotein

The 18 KDa phosphoprotein is apparent in freshly isolated cells but its phosphorylation increases dramatically after 12 hours of incubation in Ca^{2+}. A further increase in phosphorylation is seen when Ca^{2+} is removed from this protein by EGTA. This might indicate that the phosphorylation of this protein is slightly inhibited by Ca^{2+} and by removing the Ca^{2+} the protein is further phosphorylated.

This phosphoprotein (18 kDa) may be involved in binding up the Ca^{2+}, it could be involved in sperm/egg recognition or could possibly be involved in fusion. Naz and Ahmad (1994) have identified several phosphoproteins that recognize and interact with the zona pellucida. The area above the plasma membrane of the mammalian egg is the zona pellucida. This glycoprotein membrane is essential for the species specific binding of sperm and it contains high concentrations of Ca^{2+} (Gilbert, 1988). One of the phosphoproteins that recognize the zona pellucida is in the molecular range of 14 - 18 kDa. It might be possible that the 18 kDa phosphoprotein in maize sperms may also be important in sperm/egg recognition before binding.

4.3.2 Is Other Protein Phosphorylation Stimulated by Ca^{2+}?

There are 4 proteins that are phosphorylated in the presence of Ca^{2+} but not in its absence. Three of these proteins appear in the molecular weight range of 57 - 66 kDa. The fourth protein appears at 84 kDa. These proteins are dependent on the presence of Ca^{2+} for their phosphorylation. These proteins could be stimulated by Ca^{2+}-CaM or by another step in an amplification pathway.

4.3.3 One Phosphoprotein Is Not Affected By The Presence Or Absence Of Ca^{2+}

One 46 kDa phosphoprotein was unaffected by either the presence or absence of Ca^{2+}. This protein may be stimulated by cAMP, cGMP or diacylglycerol. cAMP appears to activate cAMP-dependent kinase ($kinase_A$). Regulatory and catalytic subunits of $kinase_A$, have been isolated from mammalian sperm (Tash et al., 1988). The regulatory subunit of cAMP-dependent kinase is 49 kDa.

4.4 Possible Effect of Ca^{2+} On Maize Sperms

Ca^{2+} crosses the membrane of sperms, using both active and passive transport, causing an increase in cytoplasmic Ca^{2+} concentrations. This increase in Ca^{2+} may stimulate Ca^{2+} binding proteins like calmodulin and calreticulin. Ca^{2+} may also stimulate other enzymes like phospholipase A_2, phospholipase C and proteases which will aide in the fusion process. Ca^{2+} or Ca^{2+}-CaM may also stimulate kinases or phosphatases to alter the phosphorylation of regulatory enzymes. Ca^{2+} or the cascade of reactions caused by Ca^{2+} will eventually cause membrane modifications leading to or facilitating the fusion events.

4.5 Possible Roles of Ca^{2+} in Plant Fertilization

From the results of our studies are viewed in the light of many studies of mammalian fertilization, a highly speculative conclusion could be proposed about the role calcium might play during fertilization of *Zea mays*. The pollen tube travels down the silk toward the megagametophyte for approximately 16 - 16.5 hrs (Cass et al., (unpublished data)). The sperm cells remain away from the calcium rich tip of the pollen tube. High levels of Ca^{2+} were found in the first 20 μm of the pollen tube (Zhang et al., 1995). The nearest distance between the sperm and the tip of the pollen tube was approximately 89 μM (Zhang et al., 1995). When the pollen tube enters the synergid the sperm cells are released into a region of high calcium concentration. The synergids of wheat and pearl millet contain high concentrations of Ca^{2+} (as high as 10 - 40 mM) (Chaubal and Reger, 1990, 1992). Since wheat and pearl millet are in the same family as maize, it is entirely feasible that maize synergids might also contains high Ca^{2+}. Calcium enters the sperm cells inducing a cascade of reactions. These reactions could induce changes in the quiescent state, resulting in increased sperm protein synthesis. The influx of calcium could also activate enzymes (perhaps the same enzymes activated in mammalian sperm) which would facilitate fusion by causing loss of membrane integrity. At the same time the egg cell is also changing its permeability to calcium causing the uptake of calcium which aids in the fusion process. The sperms leave the synergid, one going to fuse with the egg and the other will fuse with the polar nuclei. After fusion the Ca^{2+} is sequestered into organelles or vacuoles at the bottom of the synergid. This removal of Ca^{2+} may be important for the next step in the process of growth.

In conclusion, approximately 60% of Ca^{2+} enters the sperm cell due to an ATP dependent mechanism, approximately 20% of Ca^{2+} enters by another mechanism, probably a Ca^{2+} channel and 20% of Ca^{2+} uptake is due to binding. The transporter or channel binds Ca^{2+} before it is transported across the membrane. This study also showed for the first time the presence of CaM and calreticulin in maize sperms. Their function was not elucidated in this study but similar proteins in other systems regulate the action of Ca^{2+}. This study has also shown that there is a difference in the phosphorylation pattern of sperm proteins depending on their external and internal environment.

4.6 Future Work

4.6.1 Purification Of The 18 kDa Phosphoprotein

The 18 kD phosphoprotein from maize sperms has the same molecular weight as a protein on human sperm membranes that recognizes the zona pellucida (Naz and Ahmad, 1994). It might be interesting to isolate the 18 kDa phosphoprotein and see if it resembles the human sperm protein in any way. The first determination could be done by obtaining some antibody to this protein and seeing if it will cross react with the sperm protein. This could prove interesting because the protein could be involved in the recognition and fusion process.

4.6.2 Phospholipases and Proteases

In mammals calcium stimulates phospholipase A_2, phospholipase C and proteases (Hinkovska-Galchev and Srivastava, 1992; Nagdas 1992; Fry et al 1992; Roldan and Fragio 1993). Action of these enzymes perturbs the plasma membrane which is necessary for acrosomal exocytosis. It would be interesting to look at these enzymes over the period of sperm entry to the synergid until fusion is completed. These enzymes might play a role in the fusion process or in some way activating the sperm cell to fuse with the egg.

4.6.3 Determination of Ca^{2+} Levels In Maize Synergids And Pollen Tubes

The Ca^{2+} located in the pollen tube of maize has been evaluated using CTC (Chlorotetracycline) staining. This can be used to evaluate Ca^{2+} present but it does not distinguish between bound and free Ca^{2+}. The pollen tubes should be evaluated using Fluo-3 dextran. This chemical is fluorescent and it has a 70,000 molecular weight dextran attached to it. The dextran molecule will inhibit the fluo-3 from being sequestered into organelles and give a good indication of free Ca^{2+}. Fluo-3 dextran can also be used to look at Ca^{2+} in the synergids of maize. The Ca^{2+} in the synergids of wheat and pearl millet has been looked at using X-ray microanalysis, which also does not distinguish between the bound and unbound Ca^{2+}. It might also be possible using fluo 3 dextran to look at the division of the single celled megagametophyte becoming the 8 celled egg sac. This might indicate the origin of the high amount of Ca^{2+} in the synergid. The megagametophyte may start with an unequal distribution of Ca^{2+} within the cell or the Ca^{2+} could be imported from another region of the embryo.

References

Åkerman, K.E.O., Moore, A.L. (1983) Phosphate dependent, ruthenium red insensitive Ca^{2+} uptake in mung bean mitochondria. Biochem. Biophys. Res. Comm. **114**: 1176 - 1181

Andrejauskas E., Hertel R., Marmé D. (1985) Specific binding of the calcium antagonists [^{3}H] verapamil to membrane fractions from plants. The J. Bio. Chem. **260**: 5411 - 5414

Antonelli, N.M., Stadler, J. (1990) Genomic DNA can be used with cationic methods for highly efficient transformation of maize protoplasts. Theor. Appl. Genet. **80**: 395 - 401

Ashizawa, K., Tomonaga, H., Tsuzuki, Y. (1994) Regulation of flagellar motility of fowl spermatozoa: evidence for the involvement of intracellular free Ca^{2+} and calmodulin. J. Reprod. Fert. **101**: 265 - 272

Austin, CR. (1951) The "capacitation" of the mammalian sperm. Nature **170**: 326 - 328

Bae S.J., Kitamura S., Herbette L.G., Sturtevant J.M. (1989) The effects of calcium channel blocking drugs on the thermotropic behavior of dimyristoylphosphatidylcholine. Chem. Phy. Lipids **51**:1 - 7

Bean, B.P. (1989) Classes of Calcium channels in vertebrate cells. Ann. Rev Phys **51**: 367 - 384.

Berliner, K. (1933) The action of calcium on the heart -a critical review. Am Heart J. **8**: 548 - 562

Blowers, D.P., Trewavas, A.J. (1988) Second messengers: their existence and relationship to protein kinase. in: .W.F. Boss & D.J. Morré (Eds.), Second messengers in plant growth and development, Alan R. Liss, Inc., New York, pp. 1 - 28

Brandley, B., Schnaar, R.L. (1986) Cell-surface carbohydrates in cell recognition and response. J. Leucocyte Biol. **40**: 97 - 111 (1986)

Brandt, H., Hoskins, DD. (1980). A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm J Biol Chem **255**: 982 - 987

Brownlee C. (1994) Tansley Review No. 70 Signal transduction during fertilization in algae and vascular plants. New Phytol. **127**, 399 - 423

Campbell, AK. (1983) Intracellular calcium its universal role as regulator. John Wiley and Sons Limited, Chichester. New York. Toronta. Singapore.

Cass, D.D., Jensen, W.A. (1970) Fertilization in barley. Am. J. Bot. **57**: 62 - 70

Chandra, A., Thungapathra, M., Upadhyaya, KC. (1994) Molecular cloning and characterization of a calmodulin gene from *Arabidopsis thaliana*. J. Plant Biochem. Biotech. **3**: 31 - 35

Chaubal R, Reger BJ (1990) Relative high calcium is localized in synergid cells of wheat ovaries. Sex Plant Reprod **3**: 98 - 102

Chaubal R, Reger BJ (1992) Calcium in synergid cells and other regions of pearl millet ovaries. Sex Plant Reprod **5**: 34 - 46

Charuk, J. H.M., Pirraglia, C.A., Reithmeier, R.A.F. (1990) Interaction of ruthenium red with Ca^{2+} binding proteins. Anal. Biochem. **188**: 123 - 131

Chen, F., Hayes, PM., Mulrooney, DM., Pan, A. (1994) Identification and characterization of cDNA clones encoding plant calreticulin in barley. Plant Cell **6**: 835 - 843

Chien, K.R., Abrams, J., Serroni, A., Martin, J.T., Farber, J.L. (1978) Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, Ischemic liver cell injury. J. Biol. Chem. **253**: 4809 - 4817

Chulavatnatol, M., Panyim, S., Wititsuwannakul, D. (1982) Comparison of phosphorylated proteins in intact rat spermatozoa from caput and cauda epididymis Biol. Reprod. **26**: 197 - 207

Cocucci, M., Negrini, N. (1988) Changes in the levels of calmodulin and of a calmodulin inhibitor in the early phases of radish (*Raphanus sativus*) seed germination. Plant Physiol. **80**: 891 - 894

Cohen, P. (1983) Control of enzyme activity. Chapman and Hall, London and New York

Cohen, P. (1985) The role of protein phosphorylation in the hormonal control of enzyme activity. Eur. J. Biochem. **151**: 439 - 448

Coman, DR. (1954) Cellular adhesiveness in relation to invasiveness of cancer: electron microscopy of liver perfused with chelating agent. Cancer Res. **14**: 519 - 521

Conrad, P.A., Hepler P.K. (1988) The effect of 1,4-dihydropyridines on the initiation and development of gametophore buds in the moss *Funaria*. Plant Physiol. **86**: 684 - 687

Courtot, AMJ., Feinberg, JN., Schoevaert, DA., Rainteau, DP., Weinman, SJ. (1994). Calmodulin during human sperm incorporation into hamster oocyte: An immunogold electron microscope study. Mol Reprod Dev **38**: 170 - 177

Dasgupta, S., Mills, CL., Fraser, LR. (1994) A possible role for Ca^{2+}-ATPase in human sperm capacitation. J. Reprod. Fert. **102**: 107 - 116

De Jonge CJ., Han, H-L, Mack, SR., Zaneveld LJD. (1991a) Effect of phorbol diesters, synthetic diacylglycerols, and a protein kinase C inhibitor on the sperm acrosome reaction. J. Androl **12**: 62 - 70

De Jonge CJ., Han, H-L, Lawrie H, Mack, SR., Zaneveld LJD. (1991b) Modulation of the human sperm acrosome reaction by effectors of the adenylate cyclase/cyclic AMP second-messenger pathway. J. Exp. Zool **258**: 113 - 125

Dixon, ROD (1969) *Rhizobia*. Ann. Rev. Microbiol. **23**: 137 - 158

Dolle, R., Nultch, W. (1988) Specific binding of the calcium channel blocker [^{3}H]verapamil to membrane fractions of *Chlamydomonas reinhardtii*. Arch. Microbiol. **149**: 451 - 458

dos Remedios, C.G. (1981) Lanthanide ion probes of calcium-binding sites on cellular membranes. Cell Calcium **2**: 29 - 51

Dubery, I.A., Schabort, J.C. (1987) Calmodulin from *Citrus sinensis*: purification and characterization. Phytochemistry **26**: 37 - 40

Dupuis, I., Roeckel, P., Matthys-Rochon, E., Dumas, C. (1987). Procedure to isolate sperm cells from corn (*Zea mays* L.) pollen grains. Plant Physiol. **85**: 876 - 878

Eagle, H. (1956) Nutrition needs of mammalian cells in tissue culture. Arch. Biochem. Biophys **61**: 356 - 366

Faure J-E, Digonnet C, Dumas C (1994) An *in vitro* system for adhesion and fusion of maize gametes. Science **263**: 1598 - 1600

Fersht, A. (1985) Enzyme structure and mechanism. W.H. Freeman and company, New York. pg 98 - 120

Florman, HM., First, NL., (1988) The regulation of acrosomal exocytosis. I. Sperm capacitation is required for the induction of acrosomal reaction by the bovine zona pellucida *in vitro*. Dev. Biol. **128**: 453 - 463

Florman, HM (1994) Sequential focal and global elevations of sperm intrcellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. Dev. Biol. **165**: 152 - 164

Furuya, S., Endo, Y., Osumi, K., Oba, M., Nozawa, S., Suzuki, S. (1993). Calyculin A, protein phosphatase inhibitor, enhances capacitation of human sperm. Fert. Ster. **59**: 216 - 222

Fry, M.R., Ghosh, S.S., East, J.M., Franson, R.C. (1992) Role of human sperm phospholipase A_2 in fertilization: effects of a novel inhibitor of phospholipase A_2 activity on membrane perturbations and oocyte penentration. Biol. Reprod. **47**: 751 - 759

Gilbert, S.F. (1988) Developmental biology. second edition. Sinauer Associates, Inc. Sunderland, Massachusetts

Gong, M., Yang, Z. Tsao, TH. (1993) Isolation and characterization of calmodulin and a novel calcium-binding protein calpollenin from *Pinus yunnanensis* pollen. Plant Sci. **89**: 5 - 12

Graziana A., Fosset M., Ranjeva R., Hetherington A.M., Lazdunski M. (1988) Ca^{2+} channel inhibitors that bind to plant cell membranes block Ca^{2+} entry into protoplasts. Biochem. **27**: 764 - 468

Griess, EA., Igloi, GL., Feix, G. (1994) Isolation and sequence comparison of a maize calmodulin cDNA. Plant Physiol. **104**: 1467 - 1468

Hagiwara, N., Irisawa, H., Kanegama, M. (1988) Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. J. Phys. **395**: 233 - 253

Harvey, H.J., Venis, M.A., Trewavas, A.J. (1989) Partial purification of a protein from maize (Zea mays) coleoptile membrane binding the Ca^{2+}-channel antagonist verapamil. Biochem J. **257**: 95 - 100

Hedrich, R., Schroeder, J.I. (1989) The physiology of ion channels and electrogenic pumps in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**: 539 - 569

Hepler, PK., Wayne, RO. (1985). Calcium and plant development. Annu. Rev. Plant Physiol. **36**, 397 - 439

Hernandez, EO., Trejo, R., Espinosa, AM., Gonzalez, A., Mujica, A. (1994) Calmodulin binding proteins in the membrane vesicles released during the acrosomal reaction and in the perinuclear material in isolated acrosomal reacted sperm heads. Tissue and Cell **26**: 849 - 865

Hewitt, EJ., Smith, TA. (1975) Plant mineral nutrition. The English Universities Press

Higashino, H., Suzuki, A. (1994) Changes in Ca2+ mobilization in platelets from stroke-prone spontaneous hypertensive rates. Clin. Exp. Hyper. **16**: 89 - 103

Hinkovska-Galchev, V., Srivastava, P.N. (1992) Phosphatidylcholine and phosphatidylinosiltol-specific phospholipases C of bull and rabbit spermatozoa. Mol. Reprod. Develop. **33**: 281 - 286

Hogue DL, Cass CE (1994) *Spodoptera frugiperda* (Sf9) cells express novel purine- and pyrimidine-selective nucleoside transporters. Insect Biochem Molec Biol **24**: 517 - 523

Huacuja, L., Delgado, NM., Merchant, H., Pancardo, RM., Rosado, A. (1977) Cyclic AMP induced incorporation of ^{32}Pi into human spermatozoa membrane components. Biol. Reprod. **17**: 89 - 96

Huang, J.W., Grunes, D.L., Kochian L.V. (1994) Voltage-dependent Ca^{2+} influx into right-side-out plasma membrane vesicles isolated from wheat roots: Characterization of a putative Ca^{2+} channel. PNAS USA **91**: 3473 - 3477

Huang, B.Q., Russell, S.D. (1992a) Female germ unit: organization, isolation, and function. Int. Rev. Cyt. **140**: 233 - 293

Huang, B.Q., Russell, S.D. (1992b) Synergid degeneration in Nicotiana: a quantitative, fluorochromatic and chlorotetracycline study. Sex. Plant Reprod. **5**: 151 - 155

Jablonsky, PP., Grolig, F., Perkin, J.L., Williamson, RE. (1991) Properties of monoclonal antibodies to plant calmodulin. Plant Sci. **76**: 175 - 184

Jensen, W.A., Fisher, D.B. (1968) Cotten embryogenesis: the entrance and discharge of the pollen tube in the embryo sac. Planta **78**: 158 - 183

Jensen P (1981) Seperation of metabolic and non-metabolic steps of Rb^+ uptake in spring wheat roots with different K^+ status. Physiol. Plant **52**: 437 - 441

Johannes, E., Brosnan, J.M., Sanders, D. (1991) Calcium channels and signal transduction in plant cells. Bioessays **13**: 331 - 336

Kauss H (1987) Some aspects of calcium dependent regulation in plant metabolism. Annul Rev Plant Physiol **38**: 47 - 72

Kusaka, I., Matsushita, T. (1987) Characterisation of a Ca^{2+} uniport from *Bacillus subtilis* by partial purification and reconstitution into phospholipid vesicles. J. Gen. Microbiol. **133**: 1337 - 1342

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680 - 685

Leckband, D.E., Helm, C.A., Israelachvili, J. (1993) Role of calcium in the adhesion and fusion of bilayers. Biochem. **32**: 1127 - 1140

Lindemann, CB., Kanous, KS. (1989) Regulation of mammalian sperm motility. Arch. Androl. **23**: 1 - 22

Lui, B., Du, J.Z. (1989) Purification and some physicochemical properties of calmodulin in *Brassica campestris* pollen. Acta. Biochim. Biophs. Sin. **21**: 477 - 483

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**: 265 - 275

MacRobbie, E.A.C., Banfield, J. (1988) Calcium influx at the plasmalemma of *Chara corallina*. Planta **176**: 98 - 108

Malho R, Read ND., Pais MS, Trewavas AJ. (1994) Role of cytosolic free calcium in the reorientation of pollen tube growth. Plant J **5**: 331-341

Marmé, D. (1989) The role of calcium and calmodulin in signal transduction, in: W.F. Boss & D.J. Morré (Eds.), Second messengers in plant growth and development, Alan R. Liss, Inc., New York, pp. 57 - 80

Marshall, J., Corzo, A., Leigh, R.A., Sanders, D. (1994) Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from Zea mays L. roots. Plant J **5**: 683 - 694

Michalak, M., Milner, R.E., Burns, K., Opas, M. (1992). Calreticulin. Biochem. J. **285**: 681 - 692

Miller DD, Callaham DA, Gross DJ, Hepler PK (1992) Free Ca^{2+} gradient in growing pollen tubes of *Lillium*. J. Cell Sci. **101**: 7 - 12

Miller, R.J., Fox, A.P. (1990) Voltage-sensitive calcium channels. In Intracellular Calcium Regulation (Ed.) Bronner, F. pp. 97 -138. New York: Wiley-Liss

Milner, R.E., Baksh, S., Shemanko, C., Carpenter, M.R., Smillie, L., Vance, J.E., Opas, M., Michalak, M. (1991). Calreticulin, and not calsequestrin, is the major calcium binding protein of smooth muscle sarcoplasmic reticulum and liver endoplasmic reticulum. J. Biol. Chem. **266**: 7155 - 7165

Milner, R.E., Baksh, S., Michalak, M. (1992). Calcium binding proteins in the sarcoplasmic/endoplasmic reticulum of muscle and nonmuscle cells. Mol. Cell. Biochem. **112**: 1 - 13

Mogenson, H.L (1988) Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. PNAS USA **85**: 2594 - 2597

Morocz, S., Donn, G., Nemeth, J., Dudits, D. (1990) An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryonic suspension culture. Theor. Appl. Genet. **80**: 721 - 726

Nakamura, M., Moriya, M., BaBa, T., Michikawa, Y., Yamanobe, T., Arai, K., Okinaga, S., Kobayashi, T. (1993) An endoplasmic reticulum protein, calreticulin, is transported into the acrosome of rat sperm. Exp. Cell Res. **205**: 101 - 110

Nagdas, S.K. (1992) Bovine epididymal sperm proacrosin-acrosin system: quantification and partial characterization. Andrologia **24**: 171 - 178

Nash, P.D., Opas, M., Michalak, M. (1994) Calreticulin: not just another calcium-binding protein. Mol. Cell. Biochem. **135**: 71 - 78

Nayler, W.G. (1988) Calcium Antagonists Acedemic Press, Harcourt Brace Jovanovich, Publishers

Naz, RK., Ahmad, K. (1994). Molecular identities of human sperm proteins that bind human zona pellucida: nature of sperm-zona interaction, tyrosine kinase activity and involvement of FA-1. Mol. Reprod. Dev. **39**: 297 - 408

Noland, TD., Olson, GE., Garbers, DL. (1984) Protein phosphorylation of plasma membranes from bovine epididymal spermatozoa. Biol. Reprod. **31**: 185 - 194

Nowycky, M.C., Fox, A.P., Tsien, R.W. (1985a) Three types of neural calcium channels with different calcium agonist sensitivity. Nature **316**: 440 - 443

Nowycky, M.C., Fox, A.P., Tsien, R.W. (1985b) Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. PNAS USA **82**: 2178 - 2182

Opas, M., Dziak, E., Fliegel, L., Michalak, M. (1991) Regulation of expression and intracellular distribution of calreticulin, a major calcium binding protein of nonmuscle cells. J. Cell. Physiol. **149**: 160 - 171.

Parrish, JJ., Vredenburgh, WL., Lavin, CL. (1993) Increase in bovine sperm intracellular calcium (Ca_i) and pH (pH_i) during capacitation. Biol Reprod **48** (suppl. 1): 106

Pierson ES., Miller DD., Callaham DA., Shipley AM., Rivers Ba., Cresti M., Hepler PK (1994) Pollen tube growth is coupled to the extracellular calcium ion flux and intracellular calcium gradient: Effect of BAPTA- type buffers and hypertonic media. Plant Cell **6**: 1815 - 1828

Poovaiah, BW., Reddy, ASN. (1987) Calcium messenger system in plants. CRC Crit. Rev. Plant Sci. **6**: 47 - 103

Reed K.C., Bygrave F.L. (1974) The inhibition of mitochondrial calcium transport by lanthanides and ruthenium red. Biochem J **148**: 143 - 155

Rengel, Z. (1994) Effects of Al, Rare earth elements and other metals on net $^{45}Ca^{2+}$ uptake by *Amaramthus* protoplast. J. Plant Physiol **143**: 47 - 51

Rengel, Z., Elliott, D.C. (1992) Mechanism of aluminum inhibition of net $^{45}Ca^{2+}$ uptake by *Amaranthus* protoplasts. Plant Physiol. **98**: 632 - 638

Rincon, M., Hanson, J.B. (1986) Controls on calcium ion fluxes in injured or shocked corn root cells: importance of proton pumping and cell membrane potential. Physiol. Plant. **67**: 576 - 583

Ringer, S. (1882) Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. J. Physiol. (London) **3**: 380 - 393

Ringer, S., Sainsburg, H. (1894) The action of potassium, sodium and calcium salts on *Tubifex rivulorum*. J. Physiol. (London) **16**: 1 - 9

Roberts, DM., Lukas, TJ., Watterson, DM. (1986) Structure, function, and mechanism of action of calmodulin. CRC Crit. Rev. Plant Sci. **4**: 311 - 339

Roberts, D.M., Harmon, A.C. (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. **43**: 375 - 414

Roeckel P, Dumas C (1993) Survival at 20°C and cryopreservation of isolated sperm cells from Zea mays pollen grains. Sex. Plant Reprod. **6**: 212 - 216

Roeckel, P., Dupuis, I., Detchepare, S., Matthys-Rochon, E., Dumas, C. (1988) Isolation and viability of sperm cells from corn (*Zea mays*) and kale (*Brassica oleracea*) pollen grains. In: Wilms HJ, Keijzer CJ (eds) Plant sperm cells as tools for biotechnology. Pudoc, Wageningen, pp 105 - 109

Roldan, E.R.S., Fragio, C. (1993) Phospholipase A_2 activity and exocytosis of the ram sperm acrosome: regulation by bivalent cations. Biochem. Biophys. Acta **1168**: 108 - 114

Roux, L.P.F., Dubery, I.A. (1989) Purification and biochemical characterization of calmodulin from avocado fruit tissue. Phytochemistry **28**: 705 - 709

Russell SD (1991) Isolation and characterization of sperm cells in flowering plants. Annu Rev Plant Physiol Plant Mol Biol **42**: 189 - 204

Sanders LC, Lord EM (1992) A dynamic role for the stylar matrix in pollen tube extension. Int. Rev. Cyt. **140**:197 - 318

Sasaki, T., Michiko, N., Nakamura, F., Tanaka, T., (1992) Ruthenium red inhibits the binding of calcium to calmodulin required for enzyme activation. J. Bio. Chem. **267**: 21518 - 21523

Schroeder JI, Hagiwara, S (1990) Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+}-permeable channels. Proc. Natl. Acad. Sci. USA **87**, 9305 - 9309

Schroeder J.I., Thuleau, P. (1991) Ca^{2+} channels in higher plant cells. Plant Cell **3**: 555 - 559

Schumaker, K.S., Gizinski M.J. (1993) Cytokinin stimulates dihydropyridine-sensitive calcium uptake in moss protoplasts. PNAS USA **90**: 10937 - 10941

Shooter, RA., Grey, GO (1952) Studies of mineral requirements of mammalian cells. Br. J. Exp. Pathol. **33**: 98 - 103

Siebers, B., Gräf, P., Weiler, E.W. (1990) Calcium fluxes across the plasma membrane of *Commelina communis* L. assayed in a cell-free system. Plant Physiol. **93**: 940 - 947

Steer MW. and Steer JM. (1989) Pollen tube tip growth. New Phytologist **111**: 323 - 358

Tash, J.S., Krinks, M., Patel, J. (1988) Identification, characterization and functional correlation of calmodulin dependent protein phosphatase in sperm. J. Cell Biol. **106**: 1625 - 1633

Thompson, M.P., Piazza, G.J., Brower, D.P., Farrell Jr., H.M. (1989) Purification and characterization of calmodulins from *Papaver somniferum* and *Euphorbia lathyris*. Plant Physiol. **89**: 501 - 505

Thuleau, P., Graziana, A., Ranjeva, R., Schroeder, J. I. (1993) Solubilized proteins from carrot (*Daucus carota* L.) membrane bound calcium channel blockers and form calcium permeable ion channels. PNAS USA **90**: 765 - 769

Tsien, R.W, Tsien, R.Y. (1990) Calcium channels, stores and oscillations. Annu. Rev. Cell. Biol. **6**: 715 - 760

Verkleij, A.J., Post, J.A. (1987) Physio-chemical properties and organization of lipids in membranes: their possible role in myocardial injury. Basic Res. Cardiol. **82**: 85 - 91

Walz, B., Baumann, O. (1989) Calcium-sequestering cell organelles: *in situ* location, morphological and functional characterization. Topics Histochem. Cytochem. **20**: 1 - 45

Whitaker, M., Swann, K., (1993) Lighting the fuse at fertilization. Develop. **117**: 1 -12

Willemse, M.T.M. (1993) Calcium and calmodulin distribution in the tapetum of *Gasteria verrucosa* during anther development. Pl. Syst. Evol. (suppl.) **7**: 107 - 116

Wyn-Jones, RG., Lunt, OR. (1967) The function of calcium in plants. Bot. Rev. **33**: 407 - 426

Xing, T., Williams, LE., Nelson, SJ., East, JM., Hall, JL. (1994) Immunological detection and localization of a calsequestrin-like protein in red beet and cucumber cells. Protoplasma **179**: 158 - 165

Zhang G., Campenot M.K., McGann, L.E., Cass D.D. (1992a) Flow cytometric characteristics of sperm cells isolated from pollen of *Zea mays* L. Plant Physiol. **99**: 54 - 59

Zhang G., Williams C.M., Campenot M.K., McGann, L.E., Cass D.D. (1992b) improvement of longevity and viability of sperm cells isolated from pollen of Zea mays L. Plant Physiol. **100**: 47 - 53

Zhang G., Williams, C.M., Campenot M.K., McGann, L.E., Cutler, A.J., Cass D.D. (1995). Effects of calcium, magnesium, potassium, and boron on sperm cells isolated from pollen of *Zea mays* L. Sex. Plant Rep. **8**: 113 - 122